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Modulating the Innate Immune Response and Bacterial Fitness by Combinatorial Engineering of Endotoxin

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Modulating the Innate Immune Response and Bacterial Fitness by Combinatorial Engineering of Endotoxin

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2014

Dedication

To Michael, for being the kind of husband you think is just normal but most can only hope exists. Also to Harvey, for the fantastic laughs you provide.

Acknowledgements

I dreaded graduate school, thinking it was just something I had to do in order to get the necessary degree for the job I wanted. However, because of my mentor, Stephen, it has been a great experience filled with learning, training, and opportunity. His influence and attention have prepared me to stand on my own two science legs, and that is a debt I could only hope to repay by training other students equally well in the future. My career and my life benefitted greatly from my time in the Trent lab.

Multiple people have contributed to the work presented herein. I'd like to thank David Giles and Sean Carroll for their assistance with experiments, as well as George Georgiou and Marvin Whiteley for their collaboration and assistance. Indirectly, my lab members have also contributed to this work by sharing their lab expertise and reading manuscript drafts. I'm very thankful for their help and friendship.

To my committee members: thank you for your advice, difficult questions, and glowing letters of recommendation. I appreciate the time you have taken to listen to various presentations and read very long documents.

Finally, I owe much to my family. My husband, Michael, sacrificed time and opportunities to help me get here. My sisters, Sunny and Kelsey have given me great examples and encouragement. My parents, Donna and Spencer Thompson always expected us kids to do our best and follow our goals to the end of their roads, wherever they lead. I appreciate the encouragement and the excitement at any success along the way. Lastly, I want to thank my brilliant grandfather, Don Tyler, who always wants to hear research updates, counsel me on a life in science, and tell me how proud he is.

Modulating the Innate Immune Response and Bacterial Fitness by Combinatorial Engineering of Endotoxin

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The University of Texas at Austin, 2014

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Gram-negative bacteria decorate their outermost surface structure, lipopolysaccharide, with elaborate chemical moieties, which effectively disguises them from immune surveillance and protects them from the onslaught of host defenses. Many of these changes occur on the lipid A component of lipopolysaccharide, which is crucial for host recognition of Gram-negative infection. Despite its highly inflammatory nature, LPS is a molecule with remarkable therapeutic potential. Lipid A is a glycolipid that serves as the hydrophobic anchor of LPS and constitutes a potent ligand of the TLR4/MD2 receptor of the innate immune system. A less toxic mixture of mono-phosphorylated lipid A species (MPL) recently became the first new FDA-approved adjuvant in over 70 years. Whereas wild-type *E. coli* LPS provokes strong inflammatory MyD88-mediated TLR4 signaling, MPL preferentially induces less inflammatory TRIF-mediated responses. Here, we developed a system for combinatorial structural diversification of *E. coli* lipid A yielding a spectrum of bioactive variants that display distinct TLR4 agonist activities and cytokine induction. Mice immunized with engineered lipid A/antigen emulsions exhibited robust IgG titers indicating the efficacy of these molecules as adjuvants. Other types of modification to the lipid A domain, such as altering the length of the fatty acyl chains that anchor LPS to the cell membrane, were found to affect bacterial fitness but not drastically influence detection by the TLR4/MD2 receptor. Overall, this combinatorial approach demonstrates how engineering lipid A can be exploited to generate a spectrum of immunostimulatory molecules for vaccine and therapeutics development as well as for a deeper understanding of bacterial membrane biogenesis.

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Chapter 1: Introduction[1]

This introductory chapter includes a description of lipid A and the regulatory mechanisms controlling lipid A modification along with a discussion of the impact that these modifications have on pathogenesis, bacterial physiology and bacterial interactions with the host immune system. This information is then followed by background for the engineering of lipid A for therapeutic use.

1.1 Remodeling of the Gram-negative outer membrane

1.1.1 The Gram-negative cell envelope.

The bacterial cell envelope is a complex structure that protects the cell from the surrounding environment. A defining feature of Gram-negative bacteria is the presence of an outer membrane, which is an asymmetric bilayer with glycerophospholipids confined to the inner leaflet and lipopolysaccharide (LPS) anchored to the outer leaflet[1] (Fig. 1a,b). Similarly to most cell envelope components, LPS is made at the cytoplasmic face of the inner membrane and must be transported across the two bilayers and periplasm to become integrated in the outer membrane[1].

[1] Large portions of this chapter have been previously published. Needham, B. D. & Trent, M. S. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. *Nat. Rev. Microbiol.* **11,** 467–481 (2013).

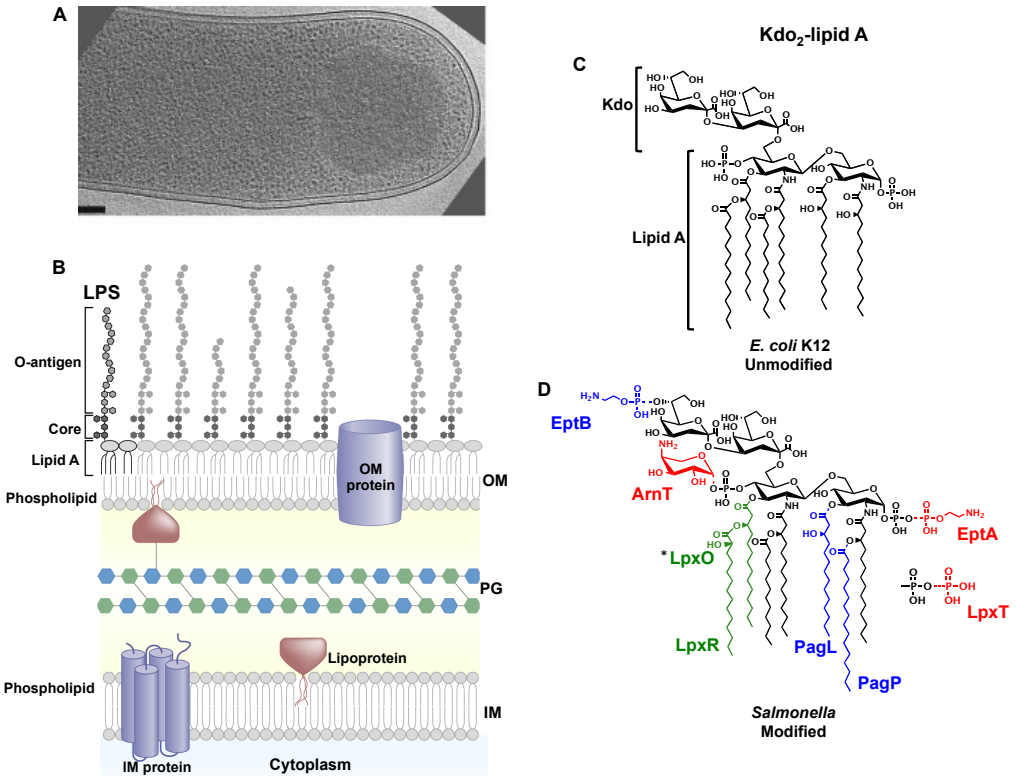

Figure 1.1 The cell envelope of Gram-negative bacteria

(a) A cryotomography image of an *E. coli* cell shows characteristic inner and outer membranes (scale bar of 200 nm). (b) Schematic of the Gram-negative cell envelope illustrating the typical membrane composition, with the outer leaflet of the outer membrane being composed of lipopolysaccharide (LPS), anchored to the membrane by its lipid A domain. Envelope components are abbreviated: outer membrane (OM), inner membrane (IM), and peptidoglycan (PG). (c) The lipid A and inner core (Kdo) portion of LPS can be extensively modified after synthesis of the β-1′,6-linked disaccharide of glucosamine that is both phosphorylated and fatty acylated (black). Modifications controlled by the PmrAB two-component system and the enzymes responsible in *Salmonella* spp. lipid A are displayed in red as follows: L-Ara4N (aminoarabinose) addition (ArnT), phosphoethanolamine addition (EptA) and phosphorylation (LpxT). Modifications controlled by the PhoPQ two-component system are indicated by blue as follows: phosphoethanolamine addition (EptB), deacylation (PagL) and acylation (PagP). Modifications enzymes not known to be controlled by a two-component system are indicated in green as follows: hydroxylation (LpxO) and deacylation (LpxR). LpxO transcription is modestly induced by PhoPQ but LpxO activity occurs independent of these two-component systems.

LPS is composed of three domains: a lipid A hydrophobic anchor, core oligosaccharide and O-antigen[2] (Fig. 1b). Some organisms (for example, *Neisseria* spp.) produce lipooligosaccharide (LOS), in which the repeating O-antigen domain is absent and is replaced by an extended core region[3]. Lipid A, the endotoxic portion of LPS and the site for many LPS modifications, is initially synthesized as a β-1′,6-linked disaccharide of glucosamine that is both phosphorylated and fatty acylated (Fig. 1c). In some organisms, such as *Escherichia coli* K12, this structure represents the typical form of lipid A in the outer membrane. Lipid A biosynthesis is well-conserved across Gram-negative bacteria and occurs via the nine-step enzymatic Raetz pathway[2].

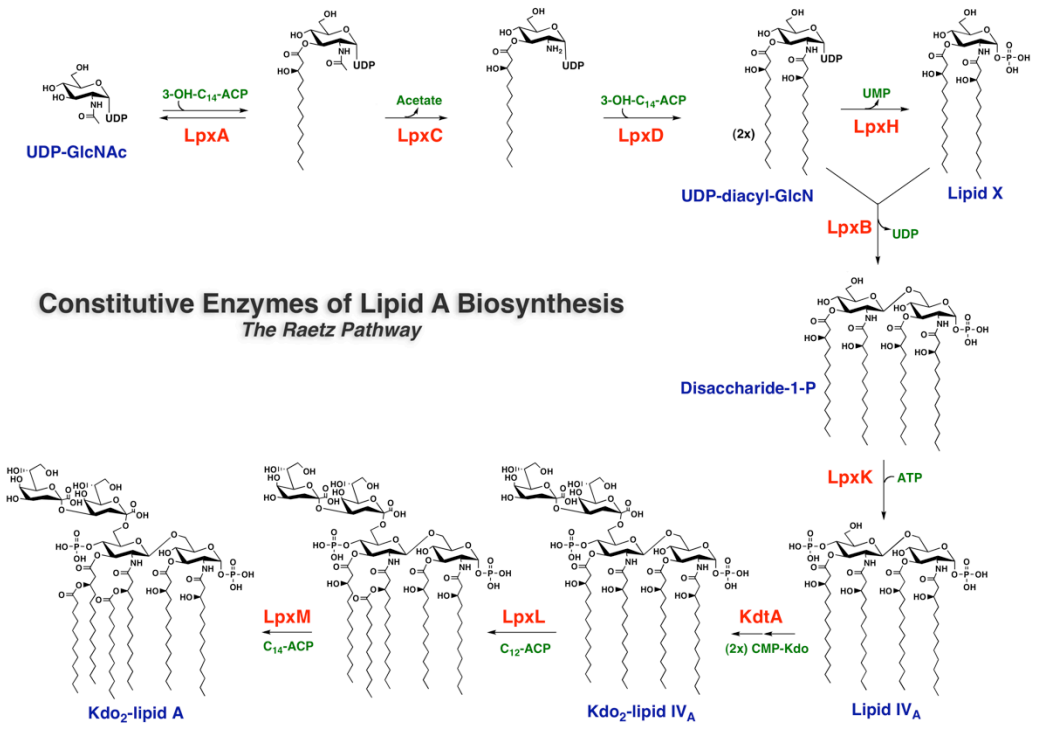

Figure 1.2 Raetz pathway of lipid A biosynthesis in *E. coli*.

Biosynthesis of the well-conserved, Kdo2-lipid A molecule via a nine-step enzymatic pathway in *E. coli*. The structures of biosynthetic intermediates are shown in black and labeled in blue. The enzyme names are shown in red. Particularly relevant to this work are the late acyltransferases, LpxL and LpxM, which will be discussed further in Chapter 3.

Despite initial studies reporting that lipid A could be modified with polar substituents (such as amino sugars[4]), it was nevertheless viewed as a static structure. This view changed in the late 1990s following characterization of the lipid A biosynthesis pathway, which established a foundation for studies that discovered that lipid A is altered post-synthesis[5–7]. In fact, Gram-negative organisms have evolved several LPS modification strategies that allow them to adapt to their unpredictable and often hostile surroundings[2]. During and after trafficking to the cell surface, lipid A can undergo extensive remodeling, resulting in the wide variety of lipid A structures that are observed across species. This is accomplished by diverse lipid A modification enzymes that remove or add acyl chains and phosphate groups, as well as other enzymes that transfer various constituents onto the molecule, such as sugars, phosphoryl containing groups and even an amino acid, all of which can influence bacterial interactions with the host (Table 1.1). Modifications also occur on other moieties of LPS but these non-lipid A modifications are not the focus of this work.

Table 1.1 Modifications to the Kdo-lipid A domain of LPS.

Enzyme	Protein localization (Active site topology)	Pathogenic organisms*	Activity	Contributors to regulation	Effect of Modification
AlmG	Inner membrane (Cytoplasmic)	*Vibrio*	*Glycine transfer to hydroxyl group of 3' acyloxyacyl chain*	Unknown	*CAMP resistance*
ArnT (PmrK)	Inner membrane (Periplasmic)	*E. coli, Salmonella, Shigella, Pseudomonas, Yersinia, Klebsiella, P. mirabilis, Burkholderia*	Aminoarabinose addition	PmrAB ParRS, CprRS (*P. aeruginosa*)	CAMP resistance
EptA (LptA, PmrC)	Inner membrane (Periplasmic)	*E. coli, Salmonella, Vibrio, Shigella,, Neisseria, Helicobacter*	Phosphoethanolamine addition	PmrAB	CAMP resistance
EptB	Inner membrane (Periplasmic)	*E. coli, Salmonella, Yersinia*	Phosphoethanolamine transfer to Kdo	PhoPQ, sRNA MgrR	Modest CAMP resistance
EptC	Inner membrane (Periplasmic)	*Campylobacter*	Phosphoethanolamine transfer to lipid A, flagellar rod, and other substituents	Present under normal laboratory conditions	CAMP resistance, motility
FlmF1	Inner membrane (Periplasmic)	*Francisella*	Glucose, mannose addition to lipid A	Present under normal laboratory conditions	Possible role in CAMP resistance
FlmF2	Inner membrane (Periplasmic)	*Francisella*	Galactosamine addition to lipid A	Present under normal laboratory conditions	Possible role in CAMP resistance
FlmK	Inner membrane (Periplasmic)	*Francisella*	Glucose, mannose or galactosamine addition to lipid A	Present under normal laboratory conditions	Possible role in CAMP resistance, TLR4 evasion
KdkA	Inner membrane (Cytoplasmic)	*Haemophillus, Bordetella, Vibrio, Pasteurella, Actinobacillus, Shewanella*	Phosphorylation of Kdo	Present under normal laboratory conditions	Possible effect on toxin delivery
KdoH1, KdoH2 (KdhA)	Inner membrane (Cytoplasmic)	*Helicobacter, Francisella, Legionella*	Removal of outer Kdo	Present under normal laboratory conditions	CAMP Resistance
KdoO	Inner membrane, (Cytoplasmic)	*Burkhoderia, Yersinia, Acinetobacter*	Hydroxylation of Kdo	Present under normal laboratory conditions	Unknown
LmtA	Inner membrane (Cytoplasmic)	*Leptospira interrogans*	Methylation of lipid A	Present under normal laboratory conditions	Unknown
LpxD2	Inner membrane (Cytoplasmic)	*Francisella*	Addition of shorter (C16) primary acyl chains to lipid A	Cold temperature	Increased membrane fluidity
LpxE	Inner membrane (Periplasmic)	*Francisella, Helicobacter, Porphyromonas,*	1-phosphatase	Present under normal laboratory conditions	CAMP resistance, TLR4 evasion
LpxF	Inner membrane (Periplasmic)	*Francisella, Helicobacter, Porphyromonas, Leptospira*	4'-phosphatase	Present under normal laboratory conditions	CAMP resistance, TLR4 evasion
LpxO	Inner membrane (Cytoplasmic)	*Salmonella, Klebsiella, Pseudomonas, Bordetella, Leigionella*	Hydroxylation of lipid A acyl chains	Present under normal laboratory conditions	Stress response coordination
LpxP	Inner membrane (Cytoplasmic)	*E. coli, Yersinia, Salmonella, Legionella*	Alternative biosynthetic acyltransferase in cold	Cold temperature	Membrane integrity and fluidity
LpxR	Outer membrane (Extracellular)	*Salmonella, E. coli 0157:H7, Helicobacter, Vibrio, Yersinia*	3'-*O*-deacylase	sRNA MicF, aminoaraminose lipid A modification	TLR4 evasion
LpxT	Inner membrane (Periplasmic)	*E. coli, Salmonella, Yersinia*	Phosphorylation of lipid A, recycling of und-pyrophosphate	PmrAB, small peptide PmrR	Unknown
PagL	Outer membrane (Extracellular)	*Salmonella, Pseudomonas, Bordetella, Burkholderia*	3-*O*-deacylase	PhoPQ, aminoarabinose lipid A modification	Lowered TLR4 activation
PagP	Outer membrane (Extracellular)	*E.coli, Salmonella, Shigella, Yersinia, Bordetella, Legionella, Pseudomonas, Erwinia, Klebsiella*	Palmitate addition	PhoPQ, membrane perturbation	Selective CAMP resistance, membrane integrity

Lipid A alterations directly impact pathogenesis by changing outer membrane permeability, promoting resistance to antimicrobial peptides and interfering with the host's ability to recognize LPS as a conserved microorganism-associated molecular pattern (MAMP)[2,8]. The diversity of LPS modification systems is quite extraordinary and the accumulated knowledge about these systems has provided deeper insight into bacterial mechanisms that contribute to human disease and immunity.

1.1.2 Regulation of lipid A remodeling

Lipid A modifications are often only necessary for a portion of the bacterial life cycle, such as host colonization, and as a consequence the enzymes responsible for the modifications are subject to both transcriptional and post-translational regulation. Many modification enzymes are embedded in the outer membrane in close proximity to lipid A, necessitating tight control for selective activity, while others are constitutively active regardless of their localization. Two-component systems, small RNAs (sRNAs), peptide feedback loops and substrate availability are all involved in directing the activity of these enzymes (Fig. 1.2).

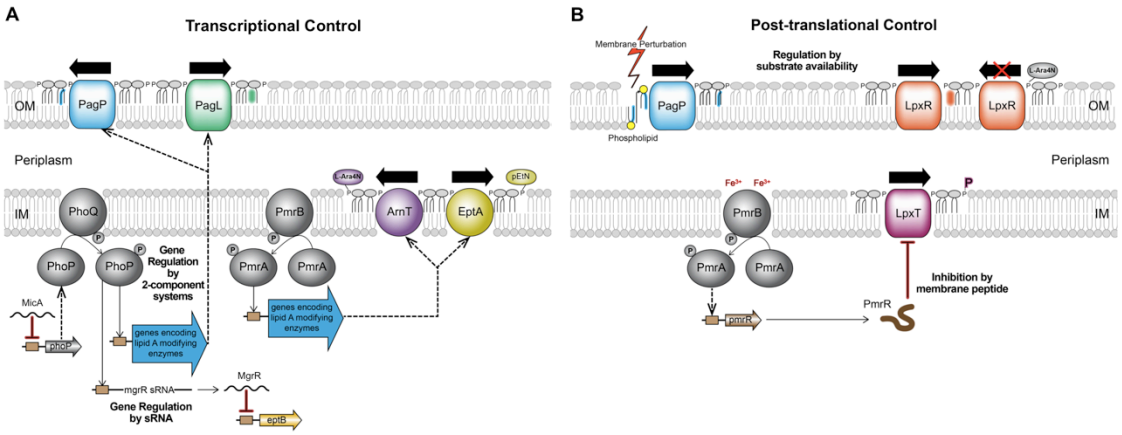

Figure 1.3 Regulation of lipid A modification enzymes.

Transcriptional (A) and post-translational (B) control is shown. Transcriptional control (A) includes gene regulation by two-component systems such as PhoPQ leading to acylation and deacylation of lipid A by the enzymes PagP and PagL, respectively, and PmrAB, leading to addition of aminoarabinose (L-Ara4N) and phosphoethanolamine (pEtN) by the enzymes ArnT and EptA, respectively. Expression of EptB, another pEtN transferase, is repressed by the small RNA (sRNA) MgrR, which is induced by PhoPQ. PhoPQ transcription is repressed by the sRNA, MicA. Post-translational control (B) includes inhibition of the kinase LpxT by the small peptide PmrR, which is upregulated by PmrAB in response to Fe^{3+} as part of a negative feedback loop that ultimately decreases the cell surface affinity for Fe^{3+} due to a less negatively charged lipid A. Also depicted in (B) is regulation by substrate availability. The acyltransferase PagP, the extracellular active site of which is normally separated from its phospholipid donor, is activated upon phospholipid displacement to the cell surface due to membrane perturbation. The deacylase LpxR is inhibited by the L-Ara4N lipid A modification, which is more prevalent in certain conditions (i.e. *Y. enterocolitica* at 21° C).

1.1.2.1 Transcriptional control

Two-component systems are typically composed of a sensor kinase that is autophosphorylated upon stimulation and transfers its phosphate group to a response regulator that serves as a transcription factor. To date, various two-component systems have been implicated in the regulation of lipid A modification enzymes, including the widespread PhoPQ and PmrAB systems, and the ParRS and CprRS systems in *Pseudomonas aeruginosa*[9–14]. Other systems, such as EvgAS, Rcs, and BvgAS, have been linked to the activity of the PhoPQ system or its downstream genes, but their direct involvement in the modification of lipid A has not yet been thoroughly investigated[15–18].

Functional PhoPQ systems are widespread among bacteria, with some variation in the activation signal used and the genes that are regulated[11,19]. Activation of the PhoPQ system in *Salmonella* spp. by acidic pH, certain antimicrobial peptides and the depletion of Mg^{2+} and Ca^{2+}, stimulates transcription of *pagP* and *pagL* and subsequent upregulation of the encoded proteins, which palmitoylate and deacylate lipid A, respectively (Fig. 1.1d, Fig. 1.2, Table 1)[10,11,20–22]. Furthermore, both of these enzymes are also subject to post-translational regulation (see below). Because the active sites of these two enzymes are found on the extracellular surface of the outer membrane, and in close proximity to lipid A, they require tight control to ensure that lipid A modification is appropriately regulated.

In *Salmonella* spp. and *E. coli* (currently unpublished in *E. coli*), PhoPQ further influences lipid A modification by activating the PmrAB system (Fig. 1.2a), although in *P. aeruginosa* the two systems have not been shown to be coupled[23]. Direct activation of PmrAB in *Salmonella* spp. occurs upon sensing iron, aluminium and low pH, and leads to the upregulation of genes such as *arnT* (also known as *pmrK*) and *eptA* (also known as *lptA* and *pmrC)* that transfer 4-amino-4-deoxy-L-arabinose (L-Ara4N, abbr.

aminoarabinose) and phosphoethanolamine groups to lipid A, respectively[12] (Fig. 1.1d, Fig. 1.2). Similar to *Salmonella* spp., the PmrAB system in *P. aeruginosa* is activated by the depletion of cations, such as Mg^{2+}, but it is also activated by antimicrobial peptides[24]. In *E. coli, Salmonella spp., P. aeruginosa* and others, the activity of the enzymes controlled by PhoPQ and PmrAB strengthen the integrity of the outer membrane permeability barrier in the presence of antimicrobial peptides and depleted cations, which enhances bacterial survival in the host[25,26].

The ParRS and CprRS are independent two-component systems that have only been found in *P. aeruginosa* and respond to subinhibitory concentrations of antimicrobial peptides[13,14]. Thus, these systems might have an important role during infection. While both systems upregulate *pmrA*, *pmrB* and the genes responsible for aminoarabinose addition to lipid A, they are differentially activated in response to some antimicrobial peptides[13,14]. For instance, the synthetic peptide CP28 activates the CprRS system, whereas the ParRS system seems to be more responsive to the peptide indolicidin[14]. The two systems react similarly to other peptides such as polymyxin B and colistin, which are both used to treat *P. aeruginosa* infections[14]. *P. aeruginosa* is a useful, but complex, model pathogen for studying lipid A modification regulatory schemes because structurally divergent forms of lipid A are associated with different types of infection, including acute bronchiectasis lung infections and chronic colonization of the cystic fibrosis lung[27].

The PhoPQ system has also been shown to mediate modification of lipid A through transcriptional activation of non-coding sRNAs. Many sRNAs modulate the expression of outer membrane proteins[28–30], but recently the MgrR sRNA of *E. coli* was shown to regulate lipid A modification[31] (Fig. 1.2a). MgrR is a transcriptional target of PhoP and is conserved in *E. coli* and certain species of *Citrobacter, Enterobacter* and

Klebsiella[31]. It regulates various genes, including the negative regulation of *eptB*[31], which encodes an enzyme that transfers phosphoethanolamine to the outer Kdo residue of LPS[8] (Fig. 1.1C). Although EptB activity results in a modest increase in resistance to the cationic antimicrobial peptide (CAMP) polymyxin B, it is unlikely that this is the main function of this enzyme because conditions that inhibit EptB simultaneously induce the other PhoPQ regulated genes, which confer higher CAMP resistance[31]. In addition to regulating an sRNA, the PhoPQ system itself is controlled by another sRNA (MicA) (Fig. 1.2a) which inhibits translation of PhoP by competitive binding to the ribosome binding site of the PhoP mRNA[32]. MicF represents another example of an sRNA that interacts with lipid A modification enzymes. This sRNA binds to LpxR transcripts, which encode a lipid A deacylase (Table 1.1, Fig 1.1c), and increases degradation of the mRNA by exposing regions susceptible to RNase E, a major contributor to RNA turnover in many bacteria[33].

1.1.2.2 Post-translational control

In addition to transcriptional regulation, lipid A modification enzymes are also subject to post-translational control mechanisms. For example, in *Salmonella enterica* serovar Typhimurium, the PmrAB system orchestrates a delayed negative feedback loop that can be activated by Fe^{3+} and allows initial uptake of the ion but sets in motion a shift in the cell surface charge that reduces Fe^{3+} retrieval from the extracellular environment. This feedback loop is established through PmrAB-induced expression of a short peptide, PmrR, which binds to and inhibits the lipid A modifying enzyme LpxT[34] (Fig. 1.2b, Table 1.1). LpxT phosphorylates lipid A (Fig. 1.1c) and thereby increases the overall net negative charge of the outer membrane. However, when LpxT activity is inhibited, other modifying enzymes are capable of transferring amine-containing constituents to lipid A[35]

and the outer membrane eventually becomes less negatively charged and has reduced affinity for Fe^{3+} [34]. This mechanism regulates lipid A modifying enzymes such as LpxT and those transcriptionally induced by PmrAB (such as *eptA* and *arnT*), prevents the intracellular accumulation of toxic Fe^{3+} concentrations, and dampens PmrA-dependent transcription in a delayed manner as a result of the lowered affinity of PmrAB-activating Fe^{3+} at the membrane. Such feedback control of lipid A modification regulatory systems by small peptides might be a common theme. In fact, other small peptides such as SafA (in *E. coli)* and MgrB (in *E. coli, S.* Typhimurium and *Y. pestis)* are inner membrane peptides that interact with the periplasmic domain of PhoQ and activate or repress PhoQ activity, respectively[36,37].

Other post-translational control mechanisms rely on substrate availability. Although the acyltransferase PagP is transcriptionally upregulated by PhoPQ in organisms such as *Salmonella spp.* and *E. coli*, its activation is enhanced by environmental or intracellular membrane stress[38]. For example, in *E. coli* PagP remains dormant in the outer membrane under standard growth conditions. However, treatment with membrane perturbing agents, such as ethylenediaminetetraacetic acid (EDTA), results in the displacement of the phospholipid donor substrate of PagP from the inner to the outer leaflet of the outer membrane, placing it in close proximity of the PagP catalytic domain[39]. PagP cleaves the phospholipid substrate, restoring the composition of the outer membrane and increasing the integrity of the permeability barrier by further acylating lipid A[40] (Fig. 1.2b).

The affinity and activity of lipid A modification enzymes are also modulated by the chemical composition of lipid A. For example, in *Yersinia enterocolitica*, LpxR deacylates lipid A at 37° C but at 21° C, LpxR activity is impeded. At 21° C, *pmrAB* and the genes necessary for aminoarabinose addition are induced and the elevated amounts of

aminoarabinose residues on lipid A seem to suppress LpxR activity[41]. In *S*. Typhimurium, PagL is similarly repressed by aminoarabinose-modified lipid A, although this effect seems to be temperature-independent[22].

These various forms of post-translational regulation afford quick response times for modification of the outer defense barrier, since the enzymes are already present and primed to function as soon as the appropriate signal is detected. Both transcriptional and post-translational regulation work together, sometimes as functionally redundant mechanisms, allowing Gram-negative bacteria to adapt to diverse environments and thereby ensure their survival. The diversity of regulatory mechanisms also illustrates the importance of lipid A structure as a major contributor to the membrane barrier.

1.2 Effects of lipid A remodeling on the innate immune response.

1.2.1 Host defenses target lipid A

Considering the intimate contact that humans have with bacteria, colonization with pathogenic Gram-negative bacteria is astonishingly low. This is largely attributed to the formidable arsenal of host defenses that eliminate invading pathogens by recognizing and responding to highly-conserved components of infectious agents, known as microorganism-associated molecular patterns (MAMPs)[42]. Because LPS is an essential component of the Gram-negative cell surface, it serves as an effective MAMP to trigger the innate immune system[1,2]. The host offers a nutrient-rich but perilous environment for a bacterium. For example, intestinal colonization requires a bacterium to journey through the acidic pH of the stomach and encounter toxic compounds such as bile and antimicrobials during transit[43], and the bloodstream is swarming with LPS-binding proteins, antibodies, complement and immune cells primed to detect LPS[1,44–47]. Every

point of entry for a bacterium is well-defended, but since many of these protective mechanisms rely on lipid A detection, the modification of lipid A affords the bacterium an opportunity to evade the immune system and establish an infection.

1.2.2 Charge-dependent binding of lipid A occurs in the host

Charge-dependent binding of various host molecules (such as CAMPs, platelet factor 4 (PF4) and members of the complement system) to the bacterial cell surface is a major contributor to host protection. CAMPs are amphipathic molecules present on mammalian mucosal surfaces, in bodily secretions (such as sweat and saliva) and in phagocytic cells. CAMP production is a highly conserved defense mechanism of most organisms, but the peptides vary widely in terms of their composition and structure. The genes encoding CAMPs are among the most rapidly evolving mammalian genes[48] and it has been hypothesized that this represents an example of co-evolution, wherein the genes are under selective pressure to mutate as a consequence of the evolution of bacterial resistance to them[49]. One of the primary mechanisms proposed for CAMP-mediated bactericidal activity is through association of the positively charged peptides with negatively charged lipid A, followed by membrane insertion and disruption of the membrane potential, leading to bacterial cell death[43,49].

PF4 is another positively charged host molecule that is conserved across vertebrates and is released upon platelet activation during infection. This molecule binds the bacterial cell surface at the lipid A domain. Antibodies specific for PF4-lipid A complexes are subsequently produced, leading to increased opsonisation and phagocytosis of the bacterium[44]. Furthermore, complement proteins also bind to lipid A and modulate the activation of the classical complement pathway to promote bacterial clearance[45–47].

1.2.3 Lipid A is detected by Toll-like receptor 4 activation

Lipid A is recognized by the Toll-like receptor 4-myeloid differentiation factor 2 (TLR4-MD2) receptor, one of many pattern-recognition receptors (PRRs) of the mammalian innate immune system. This receptor is present on a wide variety of cell types including monocytes, lymphocytes and endothelial cells[1]. Binding of TLR4-MD2 to lipid A triggers a signaling cascade that leads to inflammation, cytokine production and eventual clearance of bacteria through the recruitment of effector cells, phagocytosis, cytotoxicity and activation of the complement system[50] (Fig. 1.3). This inflammatory response can be severe, resulting in tissue damage, organ failure and death, especially in cases of sepsis[2]. Unmodified *E. coli* lipid A, which contains six acyl chains and two phosphate groups, is the strongest known TLR4 ligand, and lipid A modifications can weaken or abolish TLR4 signaling and change the nature of the downstream cytokine profile (see below)[51–54].

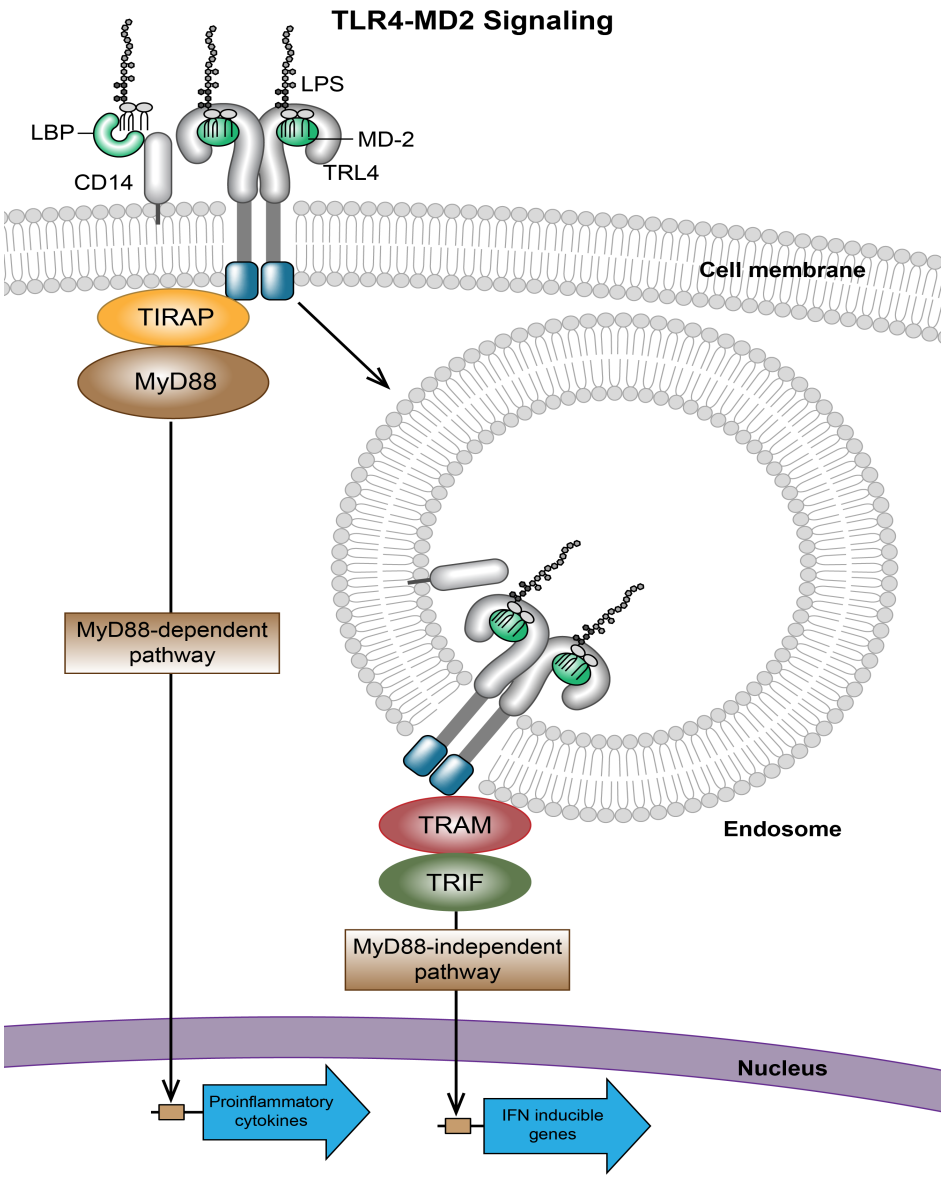

Figure 1.4 TLR4/MD2 signaling.

This simplified TLR4-MD2 signalling schematic illustrates the two responses, the MyD88-dependent and MyD88-independent (TRIF) pathways that can be differentially stimulated upon binding of modified lipid A to the TLR4-MD2 complex and lead to the production of cytokines and ultimate pathogen clearance. MyD88-dependent pathway leads to the production of inflammatory cytokines, while the TRIF pathway stimulates the expression of interferon inducible genes that are important for adjuvanticity but are less inflammatory.

Detection of lipid A by TLR4 begins with LPS-binding protein (LBP) and cluster of differentiation 14 (CD-14) binding to LPS and subsequent transfer to the TLR4-MD2 complex. This complex can then signal through two major pathways, which are named according to their adaptor proteins: Myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF)[55] (Fig. 1.3). Severe reactions to LPS are attributed to activation of the MyD88 pathway, which induces the production of proinflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and interleukin 12 (IL-12). The less inflammatory TRIF (or MyD88-independent) pathway occurs after endocytosis of the TLR4-MD2 receptor and is characterized by the production of interferon-β and interferon-inducible genes such as interferon γ-induced protein 10 (IP-10), monocyte chemotactic protein 1 (MCP1), regulated and normal T cell expressed and secreted (RANTES) and granulocyte colony-stimulating factor (G-CSF)[56]. Although it is important for mounting an optimal immune response to pathogens, the TRIF pathway does not lead to severe inflammation. Unmodified LPS from *E. coli* induces signaling through both pathways, but lipid A modifications can cause preferential recruitment of one adaptor protein over the other[57]. *In vivo*, bacterial lipid A modifications are also known to affect the potency of TLR4 activation, as described in the following section[8].

1.3 BACTERIAL EVASION STRATEGIES.

1.3.1 General evasion mechanisms

Modification of lipid A equips Gram-negative bacteria with an ability to evade immune recognition and survive within a host. First, by changing the overall charge of the bacterial surface through the addition of chemical groups to lipid A, such as

phosphoethanolamine and aminoarabinose, resistance to innate immune effectors (such as CAMPS and complement factors) increases. Second, changes in the structure of lipid A are important for bacterial pathogenesis because they directly impact recognition by the TLR4-MD2 receptor, and both the degree of lipid A acylation and phosphorylation are crucial for LPS recognition by TLR4-MD2[51]. Bacteria can remove acyl chains and phosphate groups to evade detection by this PRR or to shift the type of cytokine response induced[58]. Finally, certain modifications (such as those regulated by PhoPQ) alter the properties of the outer membrane permeability barrier, which provides resistance to harsh pH and antibiotics, among other stresses.

There are benefits and costs associated with each modification: as bacteria adapt to protect themselves against certain assaults, this may result in the loss of protection against others. For instance, PhoPQ and PmrAB-induced lipid A modifications increase resistance to CAMPS but constitutive activation of PmrAB has been shown to reduce *E. coli* resistance to the bile component deoxycholate[59]. Furthermore, PhoPQ mediated alterations have been shown to lower antibiotic resistance of *S.* Typhimurium in the presence of high Mg^{2+} concentrations[25]. However, these costs seem to be outweighed by a number of advantages of lipid A modification for bacterial virulence, which is exemplified by several pathogens including *S.* Typhimurium, *Helicobacter pylori, Yersinia pestis* and *Francisella tularensis*.

1.3.2 Evasion by *S.* Typhimurium

S. Typhimurium is an intracellular pathogen that causes gastroenteritis in humans. As a longstanding model organism for studying bacterial pathogenesis, *S.* Typhimurium provides prime examples of complex lipid A alterations (Fig. 1.1c)8. The bacterium has a diverse lifestyle and fine-tunes the composition of lipid A in response to the surrounding

environment[8,60]. During infection, *S*. Typhimurium penetrates the epithelial lining of the small intestine, invades lymphoid tissue and infects host phagocytes[61]. The unmodified lipid A synthesized by this organism is identical to that produced by *E. coli* K12 (Fig. 1.1). However, survival is promoted in the intestinal lumen and within host cells (where the bacterium encounters CAMPs, low pH and possibly other unknown signals), by activation of the PhoPQ and PmrAB systems, leading to the addition of phosphoethanolamine by EptA, aminoarabinose by ArnT and acyl chain remodelling by PagP and PagL[20] (Fig 1d). Commensal and pathogenic *E. coli* also encode these lipid A modification enzymes, but little is known about their regulation *in vivo*. In *S*. Typhimurium, another enzyme LpxO hydroxylates lipid A (Fig. 1.1d) as part of a coordinated stress response[62]. The combination of these modifications result in a remodeled outer membrane with a reduced net negative charge and increased integrity, which enhances virulence[10,26].

1.3.3 Evasion by *Helicobacter pylori*

The human stomach is the sole niche of *H. pylori*, and the organism is so well adapted to this environment that it colonizes roughly 50% of the world's population and can persist for decades[63]. To survive chronically in the host and remain undetected, *H. pylori* uses two constitutive lipid A-mediated evasion strategies: repulsion of CAMPs, (which are present at high concentrations in the gastric mucosa) and evasion of TLR4 detection (Fig. 1.4b). Similarly to most Gram-negative bacteria, *H. pylori* synthesizes a hexa-acylated lipid A, but displays only a tetra-acylated molecule lacking phosphate groups on the bacterial surface. This striking structural difference in surface-exposed lipid A arises from the action of several enzymes including dephosphorylation by LpxE and LpxF, addition of phosphoethanolamine by EptA and deacylation by LpxR (see

Table 1.1). These modifications confer resistance to polymyxin B as well as other biologically relevant CAMPs[64]. Reduced acylation and phosphorylation also lead to decreased stimulation of TLR4 and its downstream signaling cascade[51,65,66]. When these modification systems are inactivated through mutation, *H. pylori* displays hexa-acylated, *bis*-phosphorylated lipid A (Fig. 1.4b), which is a strong stimulator of TLR4[64]. The constitutive lipid A modifications on the acyl chains and phosphate groups are adaptations that allow this bacterium to persist in the harsh gastric environment amidst the several anti-bacterial components of the innate immune response.

1.3.3 Evasion by *Yersinia pestis* and *Francisella tularensis*

Y. pestis is notorious for its role in human disease throughout history, causing the Black Death plague that killed a third of the European population in the 14th century[67]. The bacterium has a complex life cycle, colonizing both the flea and human host[68]. This transition between hosts coincides with a switch in the composition of lipid A[69] (Fig. 1.4a). Inside the flea, *Y. pestis* grows at a temperature between 21-27° C and synthesizes a hexa-acylated lipid A similar to the highly inflammatory *E. coli* lipid A that strongly stimulates TLR4[70]. In humans, the bacterium encounters a temperature of 37° C and replaces this lipid A agonist with a tetra-acylated form that is a TLR4 antagonist. The tetra-acylated form of lipid A is believed to allow the pathogen to proliferate undetected in the bloodstream during the early stages of infection[71,72]. Heterologous expression of the *E. coli* acyltransferase LpxL in *Y. pestis* restores hexa-acylated lipid A and strong TLR4 stimulatory properties[71]. Strains that are only capable of producing hexa-acylated lipid A are also avirulent in mice, suggesting that deacylation of lipid A is required for TLR4 evasion and that it is crucial for *Y. pestis* pathogenesis[71].

Similarly to *Y. pestis*, the lipid A of *Fransicella tularensis* is also modified according to temperature, affecting membrane integrity and pathogenesis in its environmental vectors such as protozoa and arthropods (18-26º C) and mammalian hosts (37º C). This organism has two homologues of LpxD, an essential lipid A biosynthetic acyl transferase, which incorporate longer acyl chains at high temperature (37º C) compared to lower temperatures (25º C and 18º C)[73]. A mutant strain that is unable to produce lipid A with longer acyl chains is avirulent in mice and is also more susceptible to antibiotics and CAMPs due to increased membrane permeability[73]. These two examples of lipid A modifications emphasize that appropriate adaptation in response to temperature is important for altering the fluidity of the outer membrane, which is needed to maximize virulence of *Y. pestis* and *F. tularensis*.

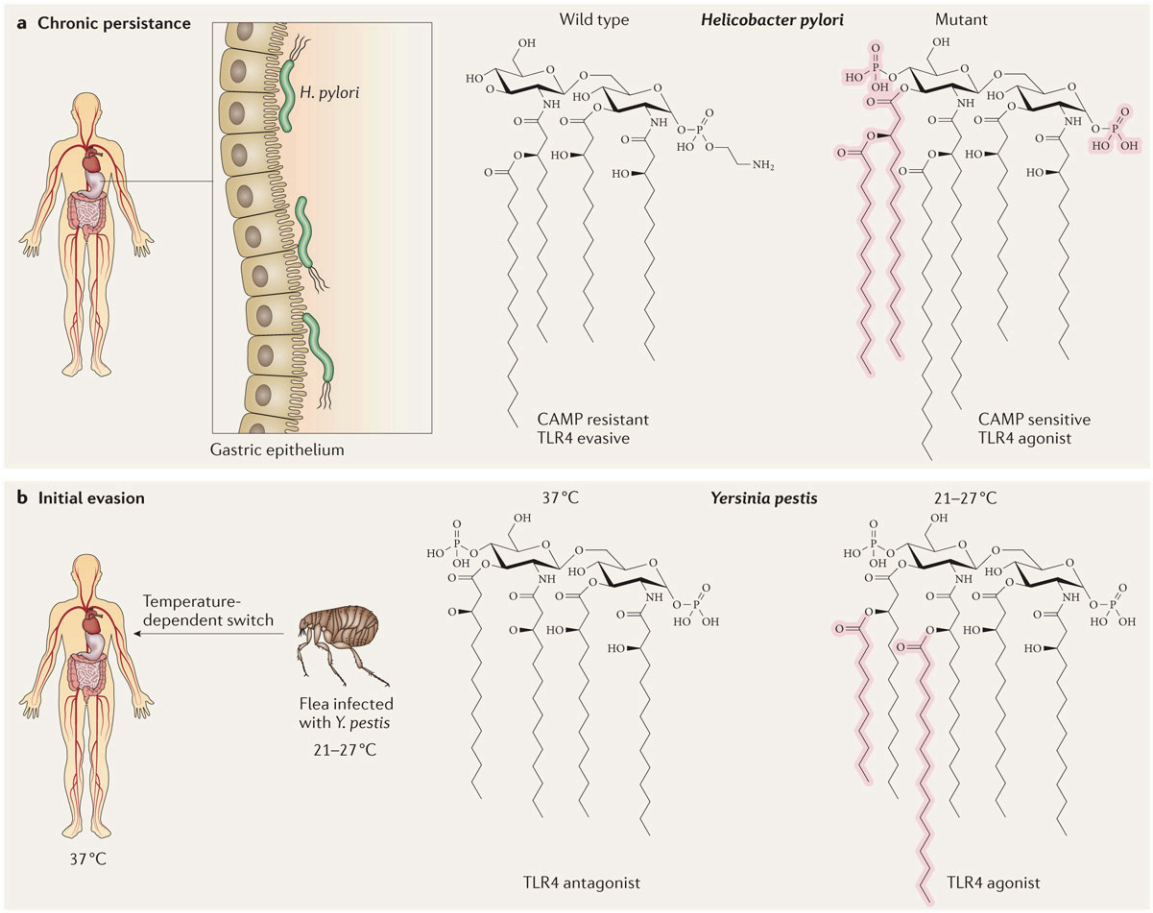

Figure 1.5 Lipid A modification strategies for survival in the host.

H. pylori and Y. pestis evolved different mechanisms of lipid A modification to aid colonization of their respective host infection sites. (A) Colonizing only one ecological niche, *H. pylori* constitutively modifies all lipid A to a form that resists CAMPs and evades TLR4. *H. pylori* lipid A is first synthesized as a hexa-acylated, *bis*-phosphorylated species by the *de novo* pathway, but through the action of five modifications a tetra-acylated form lacking the 4′-phosphate group and substituted at the C-1 position with a phosphoethanolamine is presented on the bacterial surface. Mutating *lpxE* and *lpxF* inactivates this ordered modification pathway and results in a structure resembling *E.coli* lipid A. (B) While residing in the flea-vector, *Y. pestis* produces an endotoxic, hexa-acylated lipid A. Upon transmission to the human host, the bacterium senses a shift in temperature and synthesizes tetra-acylated lipid A that escapes TLR4 detection.

1.4 LIPID A AS A TOOL FOR IMMUNE SYSTEM MODULATION

1.4.1 Monophosphoryl lipid A

Through chemical or biological modification to its phosphate groups and acyl chains, the therapeutic potential of lipid A can be harnessed while limiting the inflammatory effects of the molecule[51,55,65]. In fact, a chemically detoxified mixture of mono-phosphorylated lipid A species (MPL), derived from *Salmonella minnesota* (see figure for the structure of the predominant species, 3-*O*-deacyl-4′-monophosphoryl lipid A), recently became the first FDA-approved vaccine adjuvant in over 70 years, bringing the number of FDA-approved TLR agonists up to three[55,74]. We predict that TLR ligands are likely to be the future of vaccines and could benefit other areas as well, such as cancer research, gene therapy and bacterial production of pharmaceuticals.

Lipid A derivatives like MPL have been well-studied for their agonistic properties in terms of immune stimulation[55]. However, MPL is the only lipid A that has been tested in human cancer vaccine trials[75], and almost all studies have focused on cervical cancer, since MPL is used as the vaccine adjuvant against the oncogenic human papilloma virus (HPV)[74]. Considering the evidence that TLR4 signalling can be biased to produce certain types of cytokine responses[57], further study of other modified lipid A structures as well as chemically synthesized lipid A variants[76] could prove advantageous for development of vaccine adjuvants. To facilitate such work, we have engineered an *E. coli* library to synthesize lipid A variants with a spectrum of endotoxicity[77]. These lipid A variants have the potential to be used as components of whole cells, LPS or purified lipid A and might be used as vaccine adjuvants in the future.

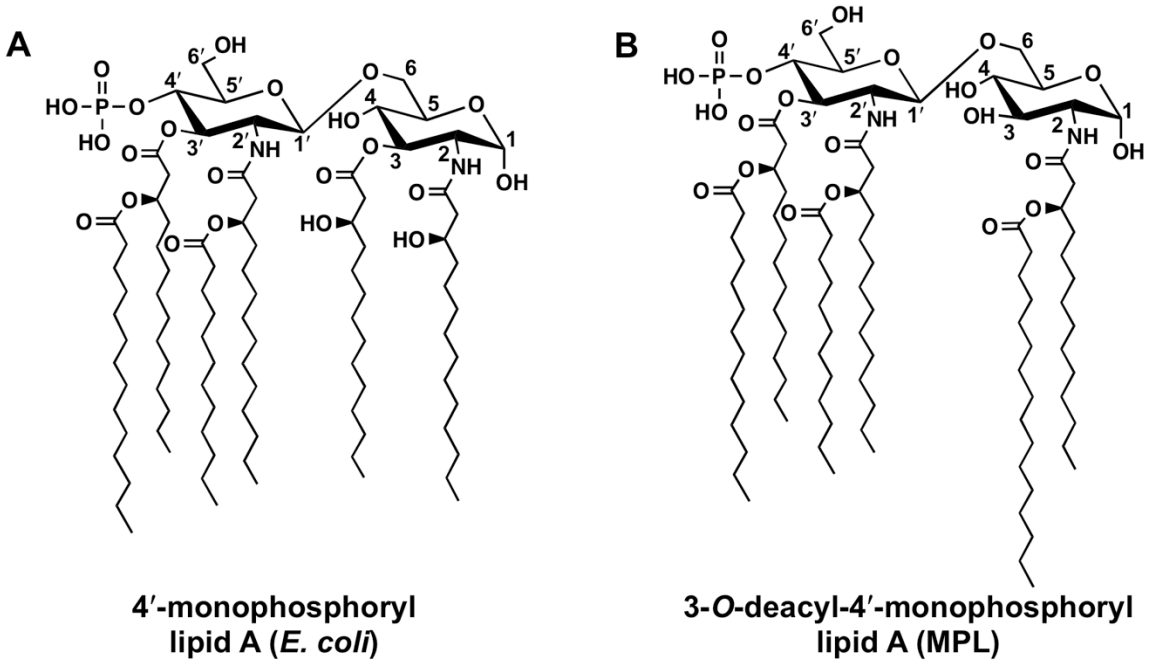

Figure 1.6 Monophosphoryl lipid A forms

(A) 4'-monophosphoryl lipid A as produced in *E. coli* expressing a 1-phophatase. (B) The structure of the predominant species in the 3-*O*-deacyl-4′-monophosphoryl lipid A (MPL) mixture that is chemically detoxified from *S. minnesota* and used as a vaccine adjuvant due to its bias toward the TRIF rather than MyD88-dependent TLR4-MD2 signalling pathways.

Whole cell vaccine strains of some organisms, such as *Salmonella* Typhimurium, have been engineered to synthesize altered lipid A structures to reduce their toxicity. Heterologous expression of an antigen from *Streptococcus pneumoniae* in such strains, provides protection against both *S*. Typhimurium and *S. pneumoniae*[52,53]. Similarly, outer membrane vesicles from *Neisseria meningitidis* with modified lipid A are now prime candidates for vaccination against *Neisseria meningitidis*[78] and other organisms, in which antigens can be heterologously expressed and targeted to the vesicle lumen or surface[79].

1.4.2 Further potential of lipid A in therapeutics

The high inflammatory response to *E. coli* is also an obstacle to gene therapy. Gene therapy strains are engineered to lyse upon phagocytosis and transfer a plasmid with mammalian expression machinery to the host[80]. For this purpose, a strain producing penta-acylated lipid A was generated to reduce the proinflammatory activity[81], but an intermediate immune response might offer an elegant balance between high phagocytosis and lowered endotoxicity. Gene therapy strains can also be engineered to colonize tumors and express tumor antigens to initiate an oncolytic response. This reaction is partly due to the lipid A/TLR4 response and the production of tumor necrosis factor[75]. In fact, lipid A immunogenicity has long been shown to be responsible for tumor regression in various models and tissue types[82].

One major hindrance to the use of purified LPS and lipid A as cancer drugs is tolerance. Tolerance develops even after one treatment, with downregulation of the immune response to LPS. Although this is detrimental to cancer treatment, it is a beneficial development for protection against sepsis for immunosuppressed patients[83]. Some lipid A analogs, such as MPL, induce less tolerance than LPS[75,84]. Direct comparison between lipid A structures could provide insight into the mechanism of

tolerance, potentially decrease tolerance for tumor regression treatments, or increase it for protection against endotoxic shock.

Tolerance has excluded MPL from cancer treatments but has not eliminated it from attention as a cancer vaccine adjuvant. In fact, although many synthetic analogs are being investigated, MPL is the only lipid A to date that has been tested in human clinical cancer vaccine trials[75]. Considering the evidence that TLR4 signaling can be biased to produce certain types of responses, other lipid A structures should be explored as well. Few studies have directly compared the effects of other lipid A structures to MPL and to LPS[55]. However, lipid A is insoluble and requires adsorption onto other adjuvants to enable delivery[55], so a modified LPS could offer more options for a soluble molecule with the lowered endotoxicity of MPL. Our combinatorial approach applied herein allows investigation into the potential for custom induction of immune responses.

The engineering of modified LPS molecules could also greatly impact subunit vaccine development. In cancer vaccines and other subunit vaccines, pathogen specific antigens are often too weakly immunogenic to be effective[55]. Thus, insufficient immunogenicity can be enhanced by lipid A or LPS adjuvants, and those antigens with intermediate immune responses can be slightly elevated by selecting LPS molecules from the library. Certain antagonistic species could also be valuable as antisepsis drugs to reduce the high number of deaths in intensive care units due to septic shock[85]. Additionally, supplementation with antagonist species of LPS has been shown to downregulate a hyperinflammatory response to some antigens or whole cell vaccines[86].

E. coli is ideal for inexpensive mass production of molecules such as DNA and protein, but LPS is a major contaminant in such pharmaceutical preparations. To purify samples within the safe clinical grade limits, many purification methods have been developed[87–89]. Unfortunately, the variety of biotechnological applications utilizing *E.*

coli makes it difficult to establish general methodologies for removal of LPS[90]. Additionally, these steps often sacrifice yield for purity, add hours to sample preparation, and require large-scale, expensive disposable supplies[91]. Using *E. coli* strains with a decreased threat of endotoxic impurity will eliminate the need for difficult purification methods.

To satisfy both pharmaceutical and therapeutic needs, lipid A would ideally be available in a spectrum of endotoxicity. Minimal endotoxicity is desirable for bacterial expression systems, whereas modest immunogenicity is more suitable for safe use in vaccines. For such diverse needs, a synthetic lipid biology approach was followed for this work in order to provide a set of *E. coli* strains that could be utilized as cells, LPS, or lipid A suitable for numerous applications.

Chapter 2: Modulating the immune response by combinatorial engineering of endotoxin[2]

2.1 INTRODUCTION

In 1892, Richard Pfeiffer introduced the revolutionary concept of bacterial endotoxin in his description of a non-proteinaceous, non-secreted toxin bound to the surface of *Vibrio cholerae*[92]. This toxin, lipopolysaccharide (LPS), is the major surface molecule of Gram-negative bacteria that triggers the host immune response during infection through recognition of the bioactive lipid A domain[2]. Lipid A, or endotoxin, is recognized by the innate immune system through the conserved pattern recognition receptor, Toll-like receptor 4/myeloid differentiation factor 2 (TLR4/MD-2) complex, which initiates a robust signal cascade leading to cytokine production that is crucial for clearance of infection but can be potent enough to result in lethal endotoxic shock[1].

The structural nature of *Escherichia coli* lipid A, with six acyl chains and two phosphate groups, is critical for complete activation of human TLR4/MD-2[51]. Many bacteria have evolved enzymes that alter their lipid A structure by modifying its number of acyl chains, phosphate groups, or polar functional groups[93]. Such modifications can alter the strength of the TLR4 response. For instance, hexa-acylated lipid A is maximally stimulatory, while tetra-acylated lipid A is antagonistic[51]. Lipid A modifications can also lead to stimulation of select TLR4 signaling pathways by mediating the recruitment of distinct sets of adaptor proteins: MyD88 (myeloid differentiation primary response gene 88), TRIF (TIR-domain-containing adaptor-inducing interferon-β), or both[55]. While

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[2] Large portions of this chapter have been previously published. Needham, B. D. *et al*. Modulating the innate immune response by combinatorial engineering of endotoxin. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 1464–1469 (2013)

strong induction of the MyD88 pathway is harmful due to high production of proinflammatory cytokines including TNF-α and IL-1β, a low level of induction is beneficial for long-lasting immunity in vaccines[55]. TRIF-mediated responses produce lower quantities of inflammatory cytokines, yet are still effective in triggering production of cytokines important for vaccine adjuvants such as IRF-3 inducible genes[56].

Efforts to understand the lipid A-TLR4 interaction have yielded improved vaccines[78]. Notably, one LPS derivative with reduced toxicity, termed MPL, has been approved to supplement an adjuvant system in vaccines worldwide[55]. MPL is a heterogeneous mixture of lipid A species from *Salmonella minnesota* R595 that has been chemically detoxified by successive acid and base hydrolysis[94]. The primary lipid A species of the MPL mixture is 3-*O*-deacyl-4′-monophosphoryl lipid A (Fig. 1.5), which induces a stronger T_H1 response than classical adjuvants such as aluminum salt precipitates (alum)[55] but unlike LPS, induces a signaling bias toward the TRIF pathway, resulting in the safe stimulation of adaptive immune responses without excessive production of inflammatory cytokines[57]. Efforts to engineer *E. coli* strains for simplified, biological production of MPL have not been successful thus far. *E. coli* strains that are dephosphorylated at the 1-position have been developed[95]; however, the acyl chain arrangement in lipid A from these strains varies structurally from the predominant *Salmonella* 3-*O*-deacyl-4′-monophosphoryl lipid A in MPL[94].

Immunomodulation by LPS variants has been recognized as a potentially advantageous therapeutic strategy for many circumstances[78,80,95]. Additionally, the lipid A modifying enzymes utilized in the present study have been individually expressed in wild type *E. coli* and/or Salmonella for proof of function[96–101], and two strains have been generated in which two coexpressed enzymes (LpxE/LpxF, and PagP/PagL, respectively) resulted in lowered antagonistic properties[102,103]. However, lipid A heterogeneity, its

essentiality to bacterial growth, the complexity of lipid A biosynthesis, and the analytical challenges posed by lipidomics have previously impeded the engineering of a single species of bacteria capable of producing nearly any lipid A species. Here, we report a system for the combinatorial engineering of *E. coli* strains that produce lipid A variants with distinct TLR4 agonist functions and a broad range of effect on the mammalian immune response (Fig. 2.1). Our method shows that coexpression of individual enzymes is not effective compared to combinatorial expression. Additionally, in many instances the engineered strains produce highly homogeneous lipid A, a feature that is particularly attractive for therapeutics development. These strains have provided a means to directly compare whole cells, intact LPS, and lipid A in the same background, eliminating confounding factors present when using different bacteria to compare lipid A structures (some not naturally found in nature). Chemical synthesis of many of these lipid A structures is complicated due to varied length of primary acyl chains and asymmetric acyl chain arrangement[104]. Also, chemical synthesis cannot be used in settings where whole bacterial cells or purified, intact LPS is required. We report on the construction and characterization of a library of 61 distinctive, engineered strains that mediate graded TLR4 dependent cytokine responses *in vitro*, and result in robust IgG titers following immunization of mice with antigen/lipid A-variant emulsions.

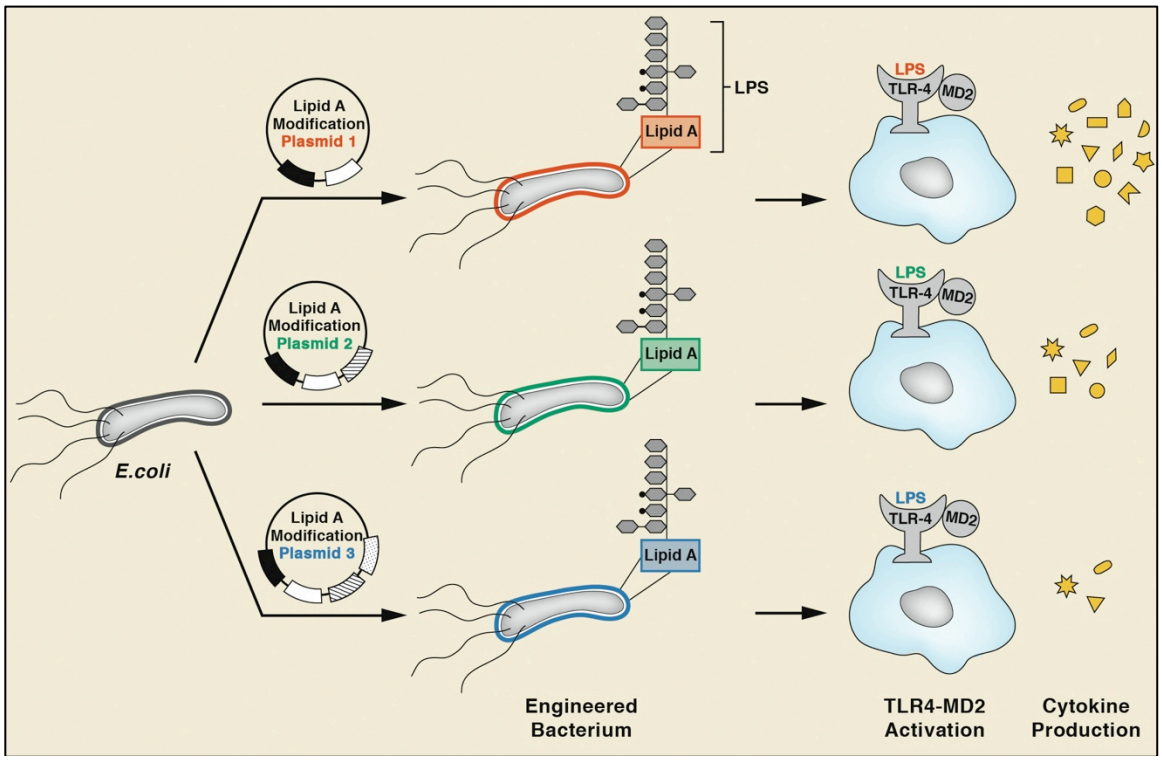


Figure 2.1 Combinatorial engineering of lipid A to generate diverse immune responses.

A schematic is shown illustrating how the outer surface of *E. coli* strains varies in LPS structure (indicated by different colors) when plasmids are expressed that contain combinations of up to five lipid A modifying enzymes. The altered LPS molecules bind and activate the TLR4/MD-2 complex differentially, altering the nature of downstream cytokine production, represented by shapes that indicate different types and quantity of cytokines released.

2.2 RESULTS

2.2.1 Combinatorial engineering of lipid A.

First, to generate an *E. coli* library with defined lipid A domains of LPS, we generated two background strains that synthesize homogeneous lipid A by deletion of the genes that modify *E. coli* lipid A under normal growth conditions (see Experimental Procedures, Table 5.1). Specifically, strain BN1 was generated from strain BN0, an *lpxT* and *eptA* double mutant. The LpxT enzyme functions to add a third phosphate group to lipid A, and when mutated the resulting lipid A is strictly *bis*-phosphorylated[105]. However, LpxT inhibition activates EptA, which adds a phosphoethanolamine to the 1-position of lipid A[35]. Mutation of *lpxT* and *eptA* increases the activity of PagP to palmitoylate the 2-acyl chain of lipid A[35], so the *pagP* gene was also deleted. As a result, strain BN1 produces hexa-acylated, *bis*-phosphorylated lipid A, the highly endotoxic, major species synthesized by *E. coli*[8]. Deletion of *lpxM* in BN1 generated strain BN2, which synthesizes only penta-acylated lipid A.

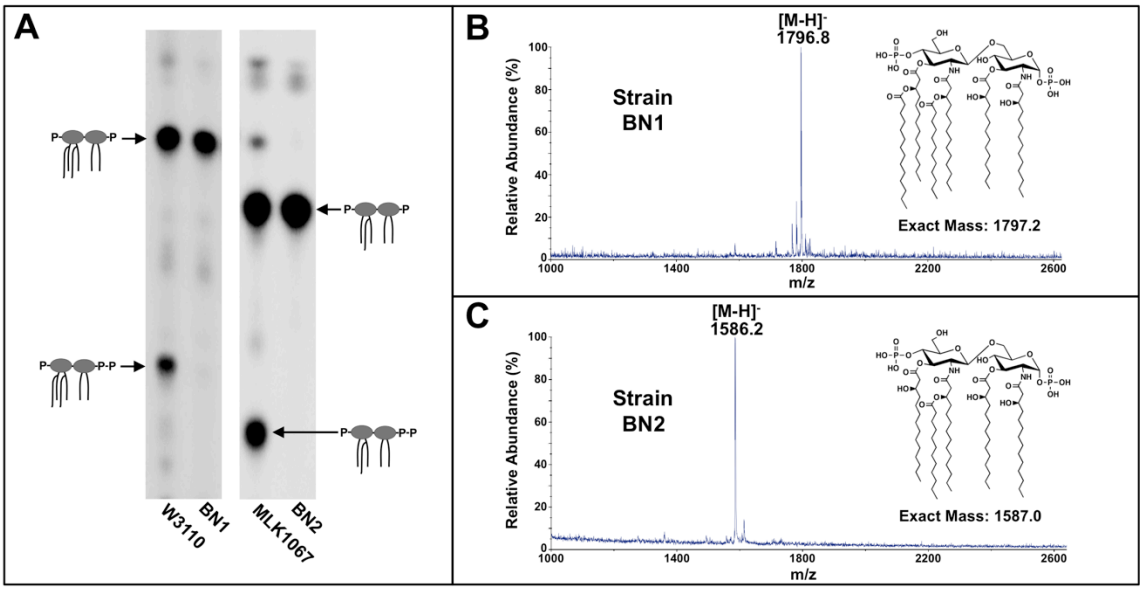
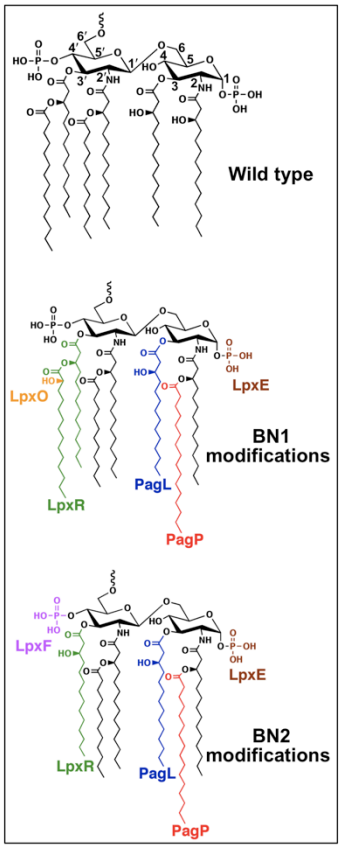


Figure 2.2 Confirmation of mutant BN1 and BN2 strains.

Radiolabeled lipid A of W3110 (*E. coli* K12), BN1, MLK1067, and BN2 was separated by TLC (a). W3110 synthesizes hexa-acylated lipid A with either two or three phosphate groups. BN1 is an *eptA, lpxT, pagP* mutant that loses the capacity to synthesize the lipid A species with three phosphate groups. These genes were deleted to eliminate modifications to the lipid A that occur under normal growth conditions[22]. MLK1067 is an *lpxM* mutant of W3110 that synthesizes penta-acylated lipid A. BN2 is an *lpxM* mutant of the BN1 strain that produces only penta-acylated, *bis*-phosphorylated lipid A. b,c) BN1 and BN2 lipid A was analyzed by MALDI-TOF MS in negative ion linear mode. Ion peaks correspond to an appropriate exact mass (±1) for BN1 hexa-acylated lipid A with two phosphates at *m/z* 1797.2 and BN2 penta-acylated lipid A with two phosphates at *m/z* 1587.0.

Thin-layer chromatography (TLC) and MALDI-TOF mass spectrometry (MS) confirmed the lipid A profiles (Fig. 2.2). BN1 and BN2 provide two distinct templates suitable for alteration by endotoxin modifying enzymes, and expression of any individual or combination of lipid A modification enzymes in these strains generates strains, distinctive in their homogeneous lipid A background. Additionally, 36 of these include combinations of enzymes that have not previously been co-expressed in any strain, which provides 61 new strains when introduced into the two backgrounds.

BN1 and BN2 were transformed with a single vector, which allows the expression of an unrestricted number of proteins[106], harboring combinations of genes encoding lipid A modification enzymes from different bacterial species, specifically PagP from *E. coli*, PagL, LpxR, and LpxO from *Salmonella enterica* serovar Typhimurium, and LpxE and LpxF from *Francisella tularensis* (Fig. 2.2b, Table 5.1)[8]. Since LpxF from *F. tularensis* is known to not function on hexa-acylated lipid A substrate[8,99], LpxF was not introduced into BN1. Additionally, LpxO from *S. enterica* hydroxylates the 3′-acyloxyacyl chain[101] that is absent in BN2, precluding its use in this strain background.



B

Enzyme	Organism source	Activity	Active site topology
LpxE	*Francisella tularensis*	Removes 1-phosphate group	Periplasmic
LpxF	*Francisella tularensis*	Removes 4′-phosphate group	Periplasmic
LpxO	*Salmonella enterica* serovar Typhimurium	Adds hydroxyl group to fatty acid myristate at 3′-position	Cytoplasmic
LpxR	*Salmonella enterica* serovar Typhimurium	Removes acyl chain(s) from 3′-position	Outer membrane
PagL	*Salmonella enterica* serovar Typhimurium	Removes acyl chain from 3-position	Outer membrane
PagP	*Escherichia coli* K-12	Adds palmitate to the primary linked acyl chain at 2-position	Outer membrane

C

Phosphorylation Pattern	Acylation Pattern: Hepta	Hexa	Penta	Tetra	Tri
Bis	BN1 pP BN1 pOP	BN1 BN1 pLP BN1 pO BN1 pLOP BN2 pP	BN2 BN1 pL BN1 pLO BN1 pPR BN1 pOPR BN2 pPR BN2 pLP	BN1 pR BN1 pOR BN2 pR BN1 pLPR BN1 pLOPR BN2 pLPR BN2 pL	BN1 pLR BN1 pLOR BN2 pLR
Mono	BN1 pEP BN1 pEOP	BN1 pE BN1 pEO BN1 pEPL BN1 pELOP BN2 pEP BN2 pFP	BN1 pEL BN1 pELO BN1 pEPR BN1 pEOPR BN2 pEPR BN2 pFPR BN2 pELP BN2 pFLP BN2 pE BN2 pF	BN1 pER BN2 pER BN1 pELPR BN2 pELPR BN2 pEL BN2 pFL BN2 pFR BN2 pFLPR	BN1 pELR BN1 pELOR BN2 pFLR BN2 pELR
None		BN2 pEFP	BN2 pEF BN2 pEFPR BN2 pEFLP	BN2 pEFL BN2 pEFR	BN2 pEFLR

Figure 2.3 Modification machinery used to generate diversified lipid A molecules in whole bacteria.

Lipid A structures of wild-type *E. coli* K12, BN1, and BN2 are shown with the names of the 6 lipid A modifying enzymes represented in color next to the group that each enzyme modifies: LpxR (green), PagL (blue), LpxE (brown), LpxF (purple) all remove the corresponding group. LpxO (orange) and PagP (red) transfer the group onto the molecule. The attachment site for remaining polysaccharide is indicated at the 6′-position of each molecule (a). The organism source, activity, and active site topology of each of the 6 enzymes (b), and the 61 combinatorial strains (c) are presented. Combinatorial strains were generated by transformation of BN1 and BN2 with a pQLinkN plasmid expressing combinations of the 6 lipid A modifying enzymes. Each enzyme is abbreviated by its final letter and ordered alphabetically in the plasmid name, i.e., LpxE is abbreviated E, LpxF is F, LpxR is R, PagP is P, PagL is L, and LpxO is O.

To confirm activity of the lipid modifying enzymes, ^{32}P-labeled lipid A isolated from the 61 engineered *E. coli* strains was analyzed by TLC, revealing 61 distinct lipid profiles, as expected. Fig. 2.3a demonstrates the variety of endotoxin species produced in BN1 and BN2 expressing combinations of lipid A modifying enzymes (Fig. 2.3a). 11 strains synthesized nearly homogeneous lipid A (Appendix), such as BN2 expressing PagL (strain BN2 pL) that produces 99.2% tetra-acylated lipid A (Fig. 2.3a). This is rare in nature, as a heterogenous mixture of lipid A species is found on the surface of most Gram-negative bacteria[8]. An additional 8 strains produce at least 75% homogeneous lipid A (Appendix), while other strains (e.g. BN2 pELPR) produce a more heterogeneous mixture as a consequence of the substrate specificity and limited expression level of the transmembrane lipid A modifying enzymes (Fig. 3a). While this heterogeneity was not our initial goal, the fact that the FDA approved adjuvant MPL is a mixture, as well as the fact that all current whole cell vaccines synthesize heterogeneous lipid A, indicated that a reproducible mixture could be just as valuable as a single uniform lipid A species. Each strain was grown and analyzed at least three times to confirm reproducibility in the lipid A profile for these mixtures.

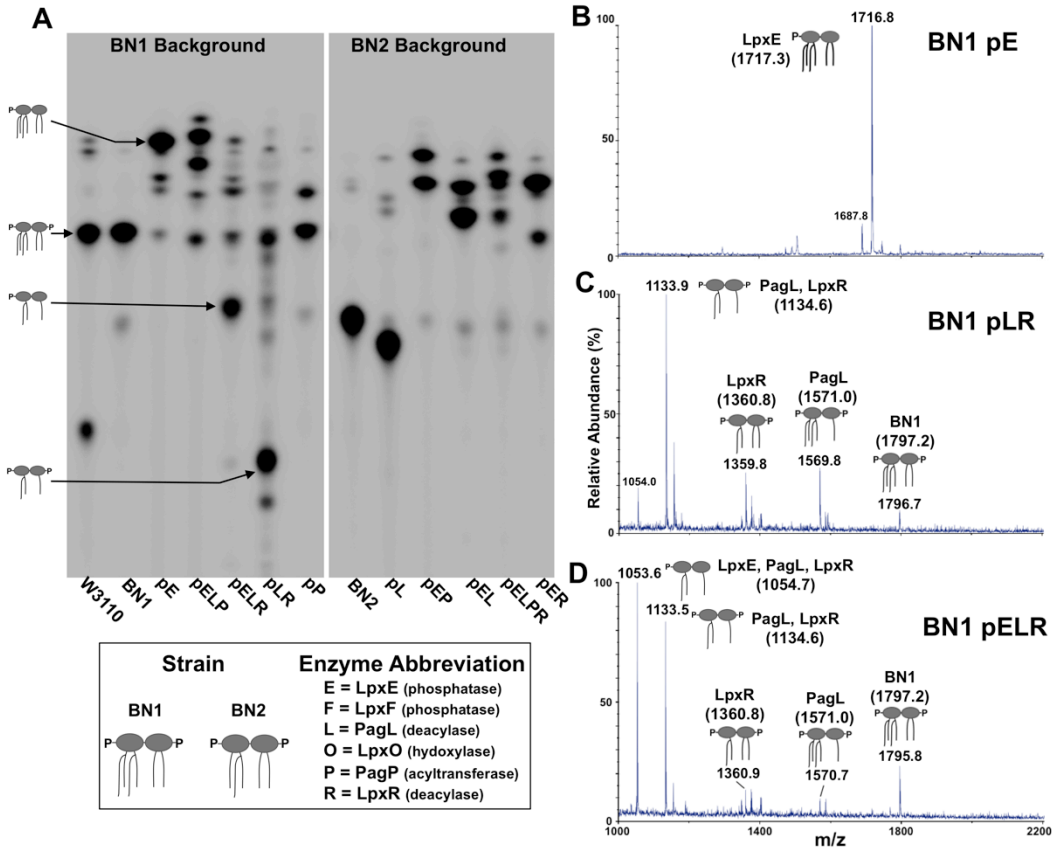


Figure 2.4 Analysis of engineered lipid A molecules.

TLC of ^{32}P labeled isolated lipid A from combinatorial strains is shown to illustrate the diversity within the collection (a). This method allows species separation, identification, and quantification based upon hydrophobicity-mediated migration. Mass spectrometry of isolated lipid A from selected strains allows further identification of lipid A species (b-d). BN1 pE produces a major peak at *m/z* 1716.8, consistent with the expected removal of one phosphate group (b). BN2 pLR produces a major peak at *m/z* 1133.9, corresponding to the mass of a tri-acylated lipid A molecule (c). This is contrasted with BN1 pELR (d), which produces a predominant peak at *m/z* 1053.6, corresponding to the dephosphorylation of the major peak seen in BN1 pLR. Minor peaks in both of these strains are similar. Peaks at *m/z* ~1360 and ~1570 correspond to masses of lipid A resulting from a single deacylation by either LpxR or PagL, respectively. The peak at *m/z* ~1796 corresponds to residual unmodified BN1 lipid A. In BN1 pLR, there is a slight loss of the labile 1-phosphate group from the major species, yielding a peak at *m/z* 1054.0.

In addition to TLC analysis, all lipid A species in the library were analyzed by MS, which allowed structural characterization based on mass and expected enzyme activities (Appendix). Figure 2.4 highlights examples of MS results for three categories of lipid A modifications: phosphate modified (b), acyl chain modified (c), or a combination of both (d). The mass spectrum of BN1 pE revealed a major peak at *m/z* 1716.8 corresponding to the removal of one phosphate group. Strain BN1 pLR yielded a major peak at *m/z* 1133.9 corresponding to a tri-acylated lipid A, resulting from deacylation by PagL and LpxR. Cells expressing LpxE, PagL, and LpxR (BN1 pELR) yield lipid A producing a major peak at *m/z* 1053.6 corresponding to tri-acylated mono-phosphorylated lipid A. These results indicate that through logarithmic growth, *E. coli* can tolerate drastic changes to its normally tightly regulated lipid A profile (Fig. 2.5).

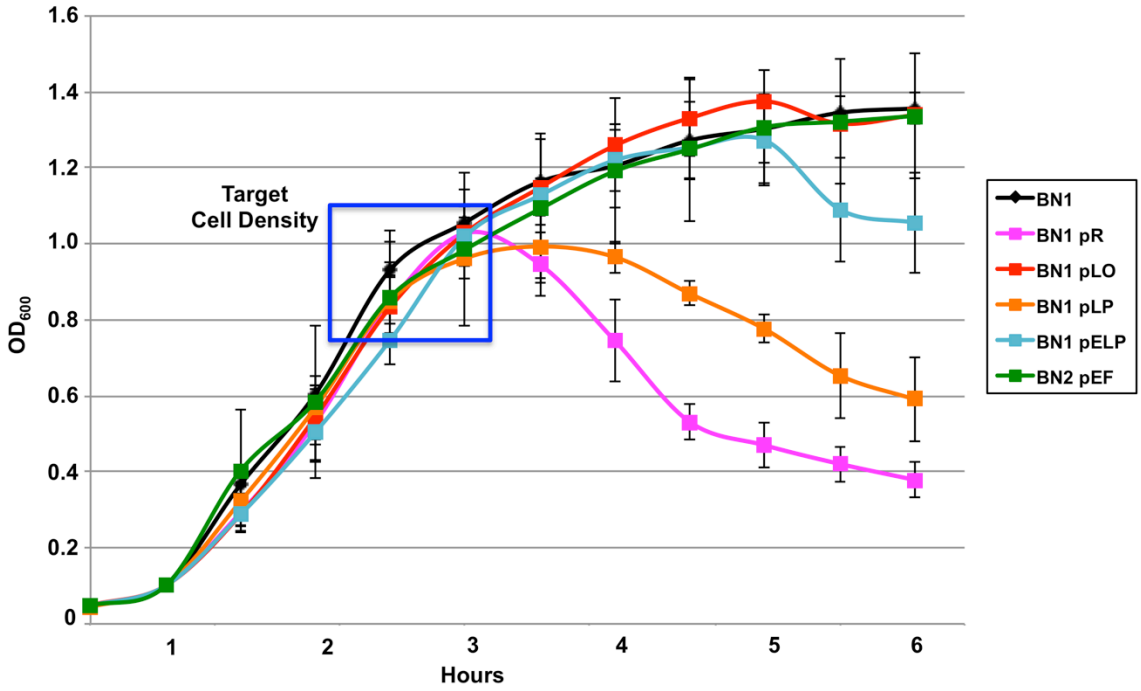


Figure 2.5 Growth curve of engineered strains.

An example graph is shown that represents the growth curve of the strains. All 61 strains reach an OD600 of at least 0.8, although some do not survive stationary phase in 96-well plate format, as represented by strain BN1 pR in purple. The background strain, BN1 is shown in black. The blue square indicates the target cell density when cells were harvested for experiments.

Coexpression of these enzymes allowed further characterization of the substrate specificity of the enzymes themselves. For example, the crystal structure of LpxR and modeling of the structure with lipid A, did not indicate any important interactions of the enzyme with the 1-phosphate group of lipid A[107]. The enzyme is Ca^{2+} dependent, and the 4'-phosphate group interacts with this cation. However, analysis of our strains that express LpxR in combination with the phosphatases LpxE and/or LpxF has shown that in the BN1 hexa-acylated background, the deacylase has increased activity (almost 100%) in the presence of LpxE, which removes the 1-phosphate group in the inner membrane before the lipid A molecule reaches LpxR in the outer membrane during synthesis and transport. In the penta-acylated BN2 background, removal of the 4'-phosphate group by LpxF completely abolishes LpxR activity, but LpxE activity does not. This is interesting since in Francisella LpxF mutants the 3'-acyl chain is retained, indicating the LpxF activity actually enhances LpxR activity in this organism[99].

2.2.2 Differential TLR4 stimulation by whole bacteria and LPS with modified lipid A.

To examine the range of TLR4 activation induced by whole bacterial cells producing diverse lipid A structures, we screened our library using HEK-Blue™ hTLR4 cells. These cells express TLR4, MD2, CD14 and the NF-kB and AP-1-dependent reporter, secreted embryonic alkaline phosphatase (SEAP). The reporter cell line was challenged with a range of colony forming units (CFU) of each of the 61 lipid A library strains. At 10^4 CFU/well, 51 of the strains displayed significantly different TLR4 responses relative to the BN1 control strain that produces wild-type lipid A ($p<0.05$). Additional controls used were the *bis*-phosphorylated, hexa-acylated W3110 parent strain and the *bis*-phosphorylated, tetra-acylated strain CMR300, which acts as a TLR4 antagonist. For direct comparison between strains, the data is presented colorimetrically

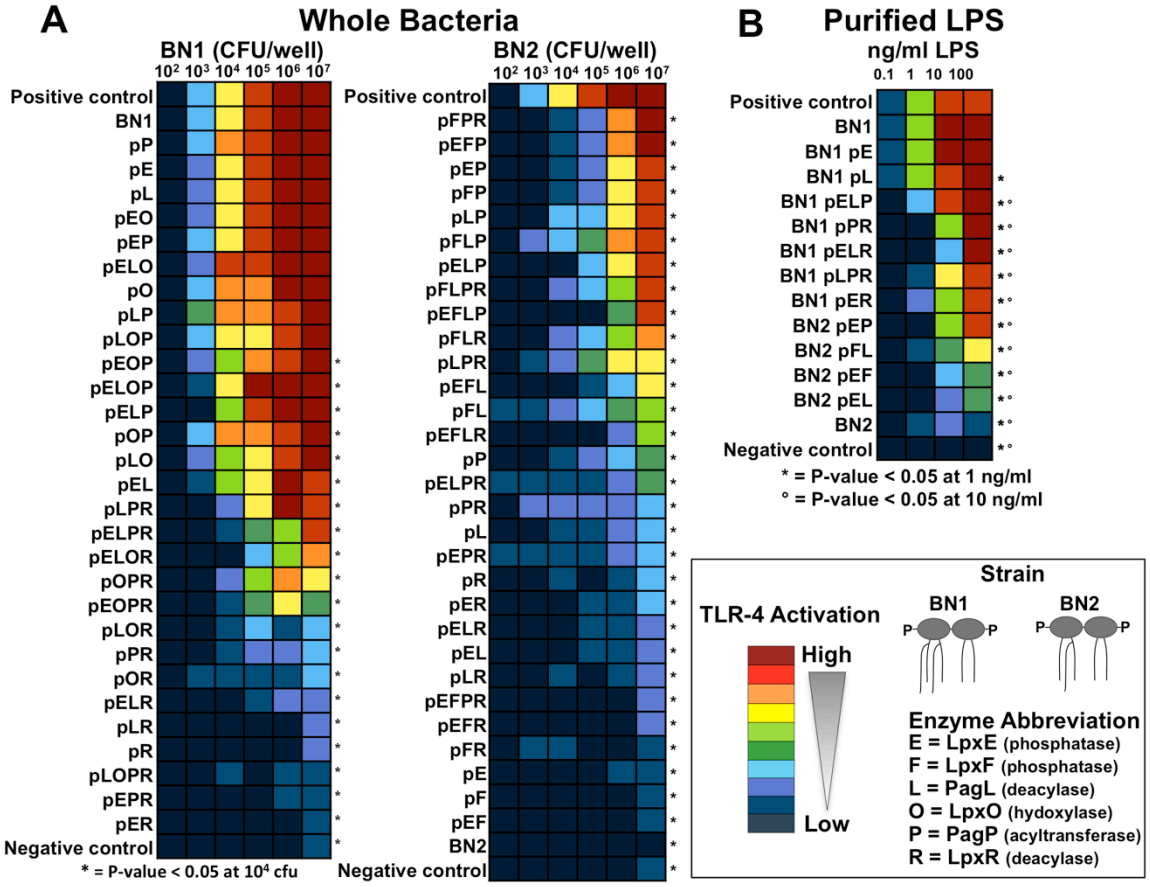


Figure 2.6 TLR4 stimulation by whole cells and purified LPS.

Stimulation of TLR4 following incubation of whole bacterial cells with HEK-Blue cells expressing TLR4, MD2, and CD14 is depicted (a). The TLR4 responses to whole cells are shown for all strains. Colors were assigned based on the TLR4 stimulation results in the BN1 strain. Rational for colorimetric designations is displayed in Fig. S3. The positive control is *E. coli* K12 strain W3110, the parent strain of the mutants used in this study. The negative control for this assay is strain CMR300, an *E. coli* strain that produces only lipid IV_A, a tetra-acylated TLR4 antagonist. The * indicates $p<0.05$ at 10^4 CFU/well. HEK-Blue-TLR4-MD2-CD14 cells were also incubated with increasing concentrations of LPS from 13 of the 61 engineered strains (b). *E. coli* K-12 LPS was used as a positive control and *R. sphaeroides* LPS, a known TLR4 antagonist, served as a negative control. The * and ° indicate $p<0.05$ at 1.0 and 10 ng/ml, respectively.

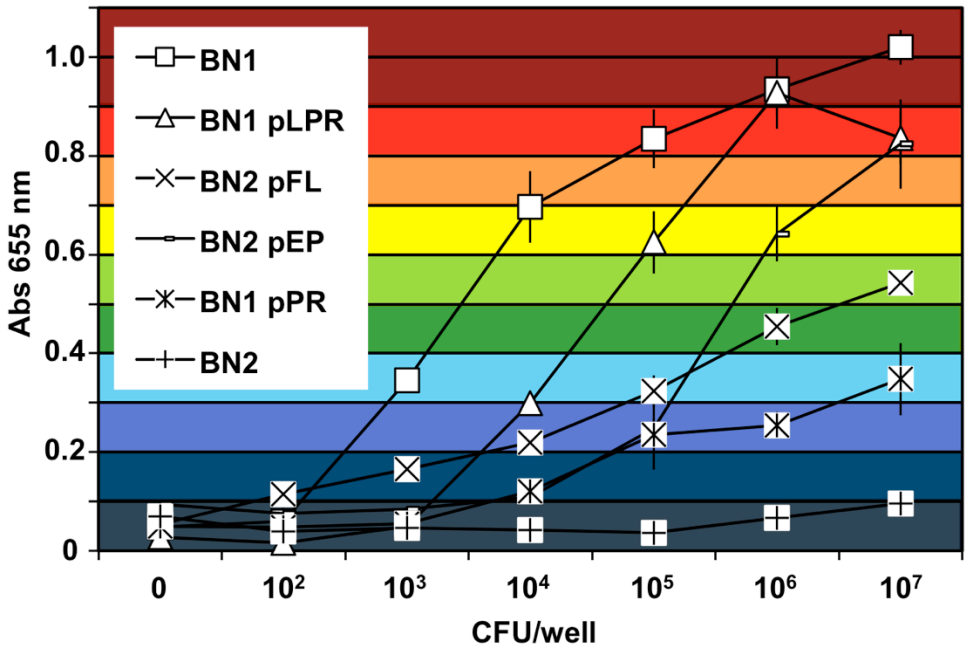


Figure 2.7 Colorimetric designations based on TLR4 stimulations by BN1.

Selected samples are shown in the graph to illustrate the range of TLR4 stimulation that results from incubation of whole bacteria cells with HEK-Blue cells expressing TLR4, MD-2 and CD14. Color scale of is based on the stimulation curve of the BN1 sample and represents the delineations of the colorimetric scale used in Fig. 2.6.

(Fig. 2.6a, Fig. 2.7), and all graphical data is supplied in Appendix X. Similar TLR4 assays were also performed using 0.1-100 ng/mL of isolated LPS from 13 of the strains (Fig. 2.6b, Appendix B). In all experiments, LPS and lipid A samples are quantified and normalized according to number of molecules. All LPS samples except BN1 pE induced significantly lower TLR4 activation than BN1 LPS at 1 ng/ml ($p<0.05$), and a similar trend was observed between cells and purified LPS (Fig. 2.6). To show full induction, pure hexa-acylated LPS was used, and *R. sphaeroides* LPS, a known TLR4 antagonist, was used as a negative control. While the effects of some lipid A modifications on LPS immunogenicity have been reported[52,103], our combinatorial approach generated a surprisingly diverse range in agonistic TLR4 stimulation and illustrated the need for further characterization of the TLR4-lipid A interaction due to the unpredictable results for many of the strains. This technique also allows investigation into reproducible mixtures of agonistic and antagonistic lipid A species within a bacterial cell that could prove optimal for adjuvant activity due to a reduction in TLR4 activation compared to wild type cells and LPS.

In this TLR4-specific assay, stimulation by BN1 bacterial cells expressing a single lipid A modifying enzyme generally did not differ from the BN1 parental strain, even in BN1 pL, in which PagL cleaves the majority of the lipid A at the 3-position to yield a penta-acylated form predicted to be less inflammatory[65] (Fig. 2.6, Appendix A). This was surprising, considering the reports from others that purified lipid A modified individually by PagL, PagP, or LpxE shows a reduction of TLR4 antagonism[55,108]. The limited alteration of the TLR4 response to whole bacterial cells expressing only one lipid A modifying enzyme validates the combinatorial approach using multiple enzymes to generate molecules with varied TLR4 activity. For example, in strain BN1 pLPR, single expression of PagL or PagP, or LpxR results in very high or very low TLR4 stimulation,

respectively. However, when all three enzymes are co-expressed, an intermediate activity is displayed (Fig. 2.6a).

We found that the TLR4 response of strains ranging from completely inactive to slightly active could be enhanced when expressed in combination. For example, in the penta-acylated BN2 background strain, expression of PagP with either or both phosphatases, LpxE and LpxF, (strains BN2 pFP, BN2 pEP, and BN2 pEFP) stimulate TLR4 almost at wild type levels at high cell counts, yet BN2 alone and each of the enzymes expressed individually in BN2 (BN2 pP, BN2 pE, and BN2 pF) are all much less stimulatory and some of them might even act antagonistically. Removal of either phosphate group from lipid A was expected to decrease the interaction with TLR4[51], but instead we see that in combination with PagP, the phosphatases act to make the strains more endotoxic. We observe a similar phenomenon in other strains, for example BN2 pLP and BN2 pFL, which each induce a higher TLR4 response than any potential subspecies within the strain (Fig. 2.6a). These results clearly demonstrate that TLR4 stimulation is substantially affected by the combinatorial expression of lipid A modifying enzymes in a manner that does not necessarily reflect the expected results from individual expression of each enzyme.

The importance of acyl chain position on the lipid A molecule during TLR4 activation has been difficult to study in the past because comparing different bacteria that synthesize lipid A with varied acyl chain position is not a controlled system. Our strains enable a direct comparison. As an example, BN2 is penta-acylated and does not stimulate TLR4 at any of the tested cell concentrations. As expected, expression of PagP (strain BN2 pP) increases activity, since it converts some of the lipid A to hexa-acylated, but not nearly as much as the highly endotoxic, wild type hexa-acylated *E. coli* lipid A. However, it is surprising that when PagL (strain BN1 pLP) is expressed, (another acyl

chain is removed and the strain then synthesizes more penta-acylated lipid A) instead of decreasing, the TLR4 stimulation increases (Fig. 2.8).

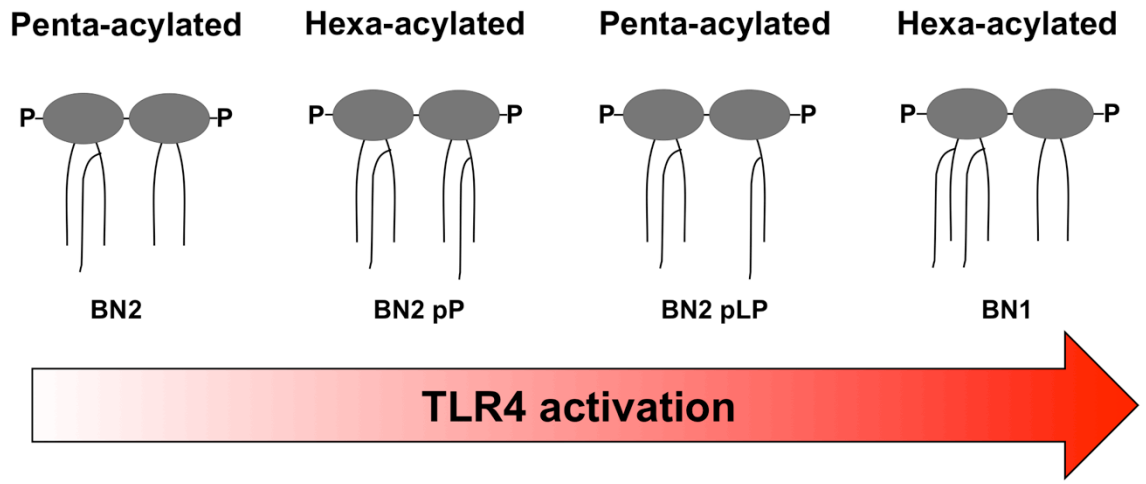


Figure 2.8 An example of the effect of acyl chain position on TLR4 activation.

BN2, which is penta-acylated, is unstimulatory. Expression of PagP in the BN2 strain increases stimulation. Including PagL expression, in BN2 pLP, further increases stimulation, even though more lipid A molecules are then penta-acylated.

2.2.1 Lipid A modification is sufficient to reduce stimulation of monocytes expressing multiple pattern recognition receptors.

Next, we investigated whether alteration of the TLR4 response through the lipid A modifications in the library are sufficient to alter the overall innate immune response, even in THP-1 (human acute monocytic leukemia) cells that also recognize many conserved bacterial patterns such as flagellar proteins, lipoproteins, and peptidoglycan in addition to LPS. To do so, bacterial cells were used to stimulate THP1-XBlue™-MD2-CD14 cells expressing all TLRs, NOD1 and NOD2 (nucleotide-oligomerization domains responsible for peptidoglycan recognition) proteins along with the SEAP reporter system that responds to stimulation of any TLR or NOD pathway. Engineered *E. coli* strains that resulted in reduced TLR4-specific activation in HEK-Blue hTLR4 cells (Fig. 2.6) also resulted in a reduced overall THP1-XBlue™-MD2-CD14 response (Fig. 2.9a), revealing that the innate immune recognition of 10^3-10^5 CFU/well of engineered *E. coli* is dominated by TLR4 activation by the lipid A moiety of LPS and not by signaling through other Toll-like receptors.

2.2.1 Cytokine profile of human monocytes stimulated by LPS.

To evaluate the cytokine profile elicited by LPS containing various lipid A modifications, we determined the concentrations of the MyD88-dependent cytokines, TNF-α, IL-6, IL-1β, and IL-8, and the TRIF-dependent cytokines, G-CSF, RANTES (CCL5), and MCP-1 (CCL2), after 24 hr incubation with 100 ng/ml LPS (Fig. 2.9b,c). A full spectrum of cytokine levels was observed, ranging from strong stimulation by LPS from the BN1 parental strain to minimal stimulation elicited by BN2 LPS. Interestingly, LPS from some strains retained the capacity to preferentially stimulate certain cytokines. For example, BN1 pLPR maintains 40% of the MCP-1 level compared to BN1, yet G-CSF production is almost completely abolished (Fig. 2.9c). Another instance of variable

production of cytokines was observed in IL-8 levels, as shown by BN2 pEP that stimulates IL-8 production equal to BN1, while all other cytokine levels were greatly diminished (Fig. 2.9b). As MCP-1 and IL-8 are important for T-cell protective immune responses, retention of their stimulation could be valuable to immune modulation, especially with the combined reduction of other inflammatory cytokines[109,110]. These results highlight how chemical modifications in LPS can impart a broad range of agonist properties to modulate cytokine production.

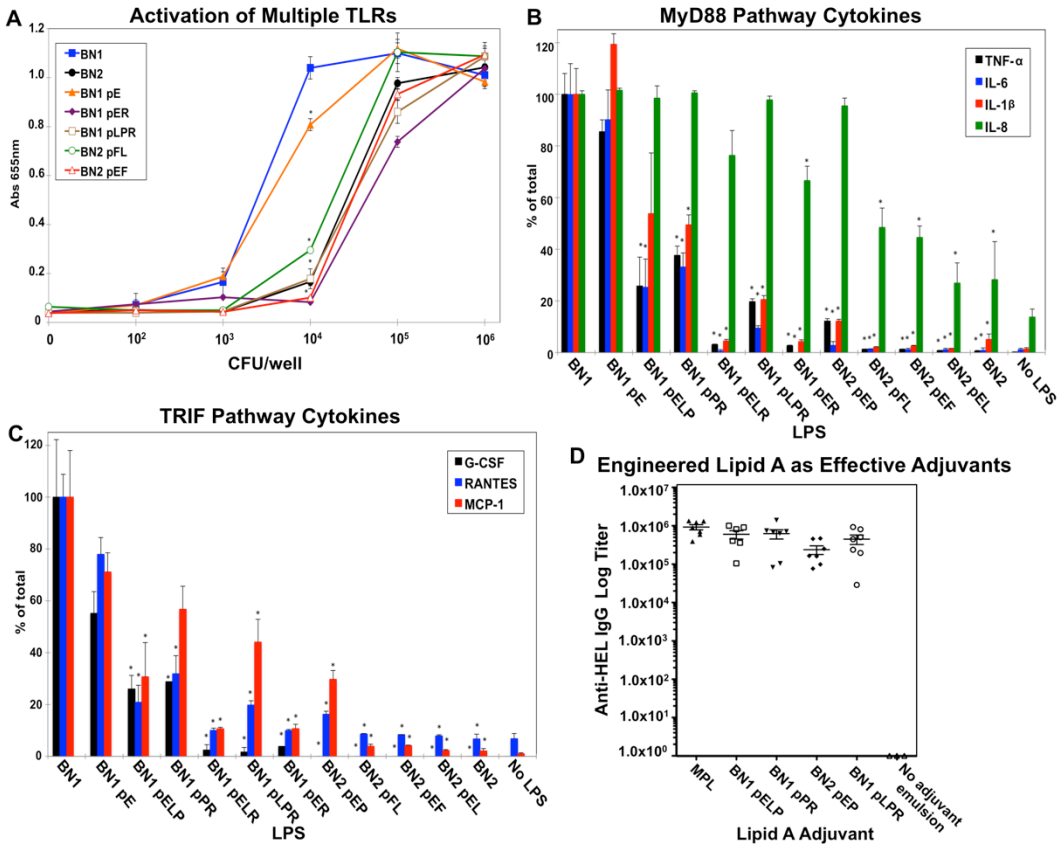


Figure 2.9 Engineered strains induce diverse stimulation and cytokine production *in vitro* and high IgG titers *in vivo*.

THP1-XBlue monocytes expressing all TLRs, Nod1, Nod2, MD-2, and CD14 were incubated with whole bacterial cells, and overall innate immune receptor activation was measured. The graph of representative samples illustrates that in the range of 10^3-10^5 CFU/well the activation of the THP1 cells was reduced, and all samples were significantly different from BN1 at 10^4 CFU/well ($p<0.001$). (b) Production of TRIF pathway cytokines (G-CSF, RANTES, and MCP-1) and MyD88 pathway cytokines (TNF-α, IL-6, IL-1β, and IL-8) by wild-type THP-1 monocytes differentiated into macrophage-like cells when incubated with 100 ng/ml LPS (b, c). Cytokine levels are presented as percent of the BN1 level. (d) BALB/cJ mice were immunized with 50 μl of an emulsion of 30 μg lysozyme from chicken egg white (HEL) with 6 pM of purified lipid A and serum was analyzed by ELISA. All lipid A adjuvants tested (BN1 pELP, BN1 pPR, BN2 pEP, and BN1 pLPR) induced a high IgG response, and only BN2 pEP was significantly lower than the MPL control ($p=0.0009$).

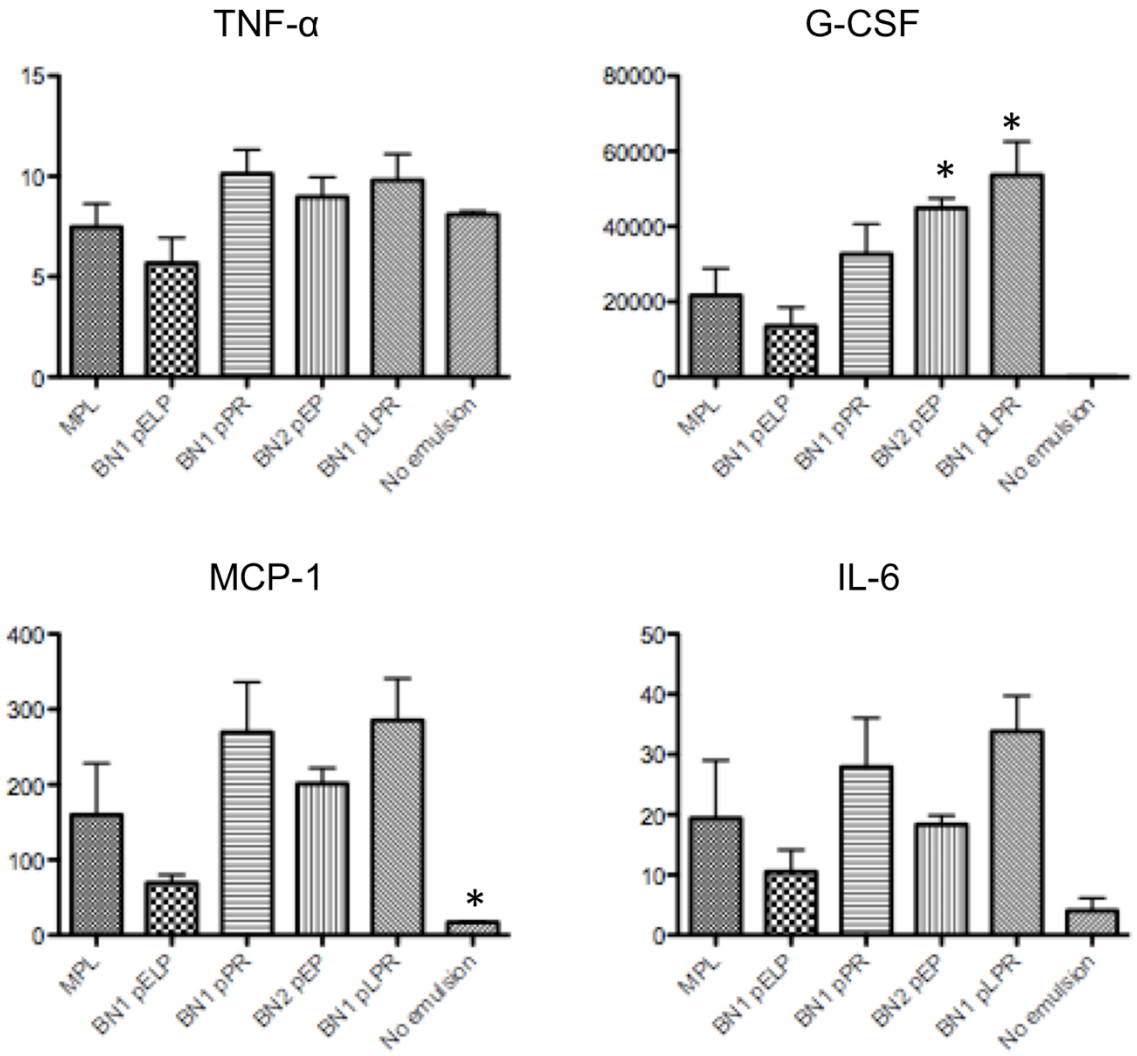


Figure 2.10 Cytokine analysis of serum from immunized mice.

Immunized mice were bled 24 hours after final immunization and cytokine levels in serum were measured by Luminex.

2.2.1 Engineered lipid A samples induce a stronge acquired immune response in mice.

To investigate the adjuvant potential of strains in this library, BALB/cJ mice were immunized with emulsions of hen egg white lysozyme (HEL) and purified lipid A from 4 strains, BN1 expressing LpxE, PagL, PagP (BN1 pELP), BN1 expressing PagP and LpxR (BN1 pPR), BN2 expressing LpxE and PagP (BN1 pEP), and BN1 expressing PagL, PagP, and LpxR (BN1 pLPR). In this experiment we used purified lipid A for direct comparison to MPL, which consists of only the lipid A domain of LPS. All tested lipid formulations resulted in high anti-HEL IgG titers, and only the BN2 pEP titer was statistically significantly lower than MPL (Fig. 2.9d). Cytokine analysis of the serum revealed a low inflammatory response measured by equivalent TNF-α levels between adjuvants, and modest variation in some cytokines such as G-CSF (Fig. 2.10). These responses in mice indicate that the combinatorial strains could facilitate biological production of safe adjuvants that are as effective as those currently used, such as MPL. Although animal models are quite useful in testing potential adjuvants, human and murine TLR4-MD2 display differential recognition of LPS. For example, tetra-acylated lipid A is an antagonist of human TLR4-MD2, but an agonist of murine TLR4-MD2[111]. This could explain the surprisingly uniform response in mice observed between lipid A samples, which can be partially attributed to lower specificity in ligand binding in the murine TLR4 compared to human TLR4, such that a wider diversity in human response to lipid A adjuvants from the library might be expected[54]. Unfortunately, a humanized mouse was not yet available during this work to compare the murine cytokine response to the adjuvant directly with human TLR4 response.

The high IgG response to lipid A from strain BN1 pELP is noteworthy because this strain produces lipid A species characteristic of the MPL mixture derived from *S.*

minnesota LPS (Appendix A, Fig. 2.11). Nonetheless, lipid A from BN1 pELP and the similar strain BN1 pELOP produce a higher percentage of the predominant species, 3-*O*-deacyl-4′-monophosphoryl lipid A found in commercial MPL preparation from *S. minnesota* (Fig. 2.11). This finding indicates that engineered *E. coli* can potentially be employed to produce high purity MPL, thus eliminating the need for extensive chemical treatment of *S. minnesota* LPS required to generate the adjuvant[94]. To ensure that purification of the 3-*O*-deacyl-4′-monophosphoryl lipid A species is feasible, we performed liquid chromatography and analyzed the purified species by MS, confirming the isolation of the species of interest (Fig. 2.11).

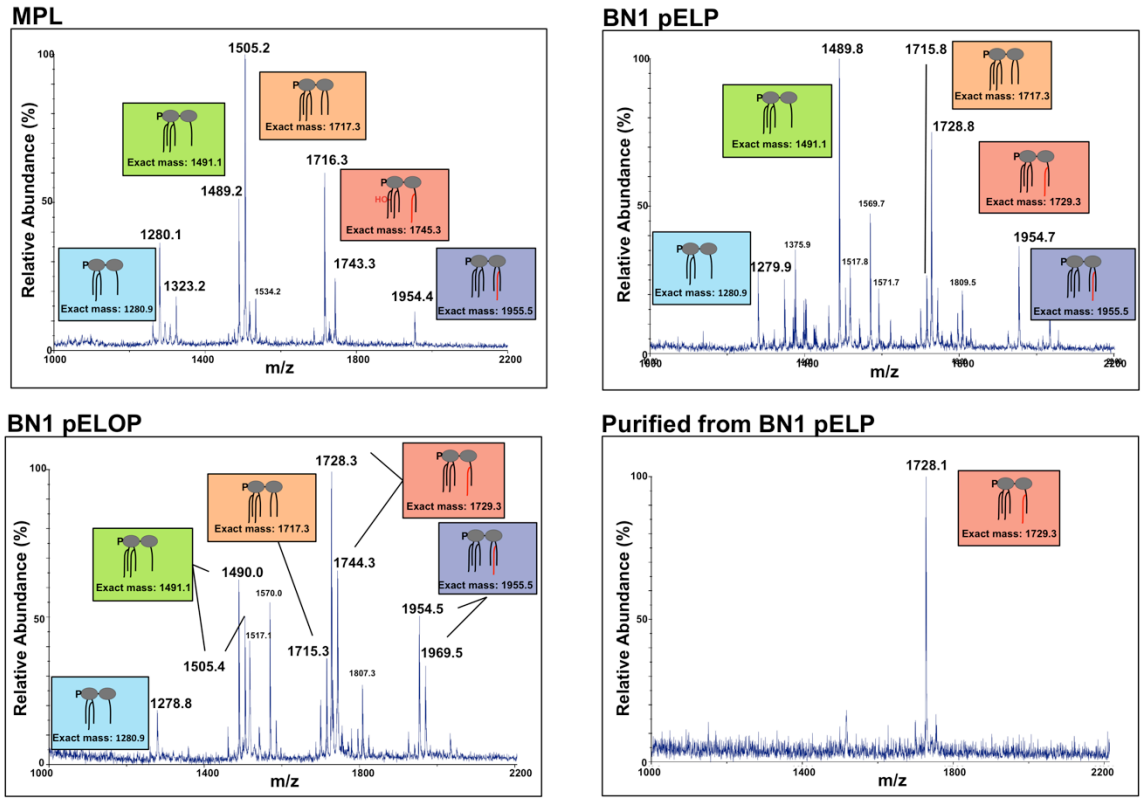


Figure 2.11 Mass spectra of engineered strains compared to MPL from *S. minnesota*.

MS data is presented of MPL from *S. minnesota*, the two strains from the library that produce similar profiles, even without additional chemical treatment, and the purified 3-*O*-deacyl-4′-monophosphoryl lipid A. Colored boxes indicate structures with the same phosphorylation and acyl chain patterns. Red box indicates the hydroxylated and nonhydroxylated 3-*O*-deacyl-4′-monophosphoryl lipid A, the most notable species of MPL.

2.2.1 Discussion

We report that combinatorial expression of lipid A modifying enzymes in *E. coli* results in an array of strains with structurally diverse lipid A species that induce differential TLR responses and cytokine profiles, many of which could not be predicted based on previous data of the TLR4-lipid A interaction. Modified LPS from these engineered strains can be utilized as (i) the major immunogenic surface component of whole bacteria, (ii) a purified LPS molecule, or (iii) free lipid A molecules following LPS hydrolysis. The effect of some lipid A modifying enzymes on TLR4 agonist activity were previously unreported. For example, we show that LpxR activity diminishes TLR4 stimulation, as expected due to reported effects of reduced acylation[65]. The results of some strains could not have been predicted based on previous work on the importance of acylation and phosphorylation of lipid A[51], e.g. BN2 pFL, which generates tetra-acylated and 4′-dephosphorylated lipid A yet consistently shows higher TLR4 stimulation than its penta-acylated, *bis*-phosphorylated parent (Fig. 2.6a). Other strains, such as the extensively modified monophosphorylated, tri-acylated lipid A of strain BN1 pELR, show that *E. coli* tolerates severe alterations in lipid A structure through removal of three of its six lipid A acyl chains and one phosphate group without apparent growth defects (Fig. 2.4a,c). This library provides a versatile tool and illustrates the utility of *E. coli* for production of modified lipid A molecules.

The diversification of modified LPS molecules that induce distinct cytokine responses offers the potential to produce new adjuvants that may be able to modulate humoral immune responses for more effective and longer-lasting protection. Although intact LPS or lipid A molecules from the library could allow pairing of adjuvants suitable for the desired response of a particular antigen, lipid A is insoluble and requires

adsorption onto alum to enable delivery[55]. Thus, detoxified whole LPS may be a more attractive soluble adjuvant.

This combinatorial library also provides tools to overcome hurdles in other aspects of industry, such as DNA and protein expression, gene therapy, and drugs for anti-sepsis or cancer. *E. coli* is ideal as an inexpensive, high-level expression strain, but LPS is a major contaminant in such preparations. Using *E. coli* strains with a diminished threat of endotoxic impurity could improve the safety of these preparations. Altered immunogenicity of whole *E. coli* cells through combinatorial diversification of lipid A would also improve gene therapy strains. Bacteria can be engineered to present antigen in tumors or invade particular tissues to transfer genetic material to a host, and a strain that selectively modulates the immune response could provide an optimal vector for such mechanisms[80,95]. In addition to lowered toxicity important for expression and gene therapy strains, some lipid A species from the library could possess antagonistic properties that inhibit TLR4 signaling. As antisepsis drugs, they could serve to block the strong inflammatory response during to septic shock[85]. In this work, we have followed a synthetic lipid biology approach to generate a combinatorial library of lipid A molecules to satisfy a wide array of biotechnological and therapeutic needs, the scope of which will continue to broaden as further investigation into the potential of these strains is completed.

Chapter 3: Determining the effects of altering the secondary acyl chain length of *E. coli* lipid A

3.1 INTRODUCTION

Gram-negative bacteria are equipped with mechanisms to modify their protective outer membrane in order to adapt to hostile and dynamic environments[12]. Yet certain aspects of the outer membrane barrier remain strictly uniform[34]. The major exterior molecule of the outer membrane, lipopolysaccharide (LPS), displays both of these characteristics; the well-conserved, tightly-controlled, and essential LPS molecule also frequently undergoes structural remodeling[1–34]. A prominent site within LPS for this dichotomy of conservation and variation is the lipid A domain, which is consistently synthesized as a hexa-acylated, *bis*-phosphorylated glucosamine disaccharide (Fig. 3.1), but can later be modified in various ways that promote bacterial survival in harsh environments and within the mammalian host[24]. One aspect of lipid A production that has particularly rigid constancy within an organism is the length of each acyl chain of this hydrophobic anchor[4]. However, understanding the contribution of acyl chain length to membrane properties has been impeded by the difficulty in engineering the essential lipid A biosynthetic enzymes and by the confounding modifications that can occur to lipid A, post-synthesis.

Within a species, the arrangement and length of the acyl chains are consistent in each lipid A molecule, and in *Escherichia coli*, each acyl chain is 14 carbons in length with the exception of the secondary acyl chain at the C2′-position, which is 12 carbons long[3,5678] (Fig 3.1). Likewise, each organism encodes enzymes with specific carbon rulers, but these vary among species and range from a specificity for acyl chains that are 10 carbons in length up to 27 in some organisms[9]. The biosynthetic enzymes responsible for

acyl chain addition to lipid A maintain specificity by the precise length of their hydrophobic fatty acyl pocket, or carbon ruler[6,74]. Although these enzymes generate homogeneous lipid A within each cell, the size of the carbon ruler, and thus the length of the acyl chains of lipid A, differ between organisms[4,9]. The essential nature of the first 8 of 9 enzymatic steps of lipid A biosynthesis (Fig. 1.2) have made engineering the length of acyl chains within a particular organism difficult, although temperature sensitive point mutants in the primary acyltransferases have been generated to allow expression of some homologs from other organisms for enzyme characterization[10,11].

One of the principal difficulties in comparing organisms that produce lipid A with differing acyl chain length is the confounding effects of extensive modification that occurs elsewhere on the lipid A molecule post-synthesis, but differs among species. Because lipid A forms the outer leaflet of the outer membrane and anchors the polysaccharide tail of LPS to the cell surface, its integrity is crucial to protrude toxic molecules from the bacterial cell. Even slight modifications to its structure can enhance or compromise survival in a harsh extracellular environment. Loss, gain, or decoration of acyl chains can increase or decrease resistance to antimicrobial compounds up to 100-fold[12–14]. The charged phosphate groups of lipid A can be masked, modified, or removed, limiting the negative charge of the cell surface and altering the association of cationic antimicrobial peptides[151617]. Modifications such as these have been shown to affect the normally robust permeability barrier to many compounds such as detergents and many antibiotics, including aminoglycosides, macrolides, penicillins, polypeptides, glycopeptides[131819]. Prior work on the effect of lipid A acyl chain length to the permeability barrier is limited, but a temperature sensitive mutant of the primary acyltransferase, *lpxA*, was complemented with the homolog from *Neisseria meningiditis*, which resulted in a C12 at the 3 and 3′-position primary acyl chains instead of the typical

C14. The shortened chains caused an increase in susceptibility to novobiocin, erythromycin, rifampicin, and bacitracin[11]. Whether the secondary acyl chains of lipid A have similar results has not been systematically studied.

Lipid A structure is also crucial for detection and control of Gram-negative infection by the host innate immune system. The lipid A domain of LPS triggers the toll-like receptor 4/ myeloid differentiation factor 2 homodimer (TLR4/MD2), initiating inflammatory cytokine signaling pathways that ultimately lead to bacterial clearance. The acyl chains of lipid A fit snugly into the hydrophobic pocket of each unit of the MD2 cofactor within the homodimer interact with the which associates with TLR4, and a homodimer forms. The hexa-acylated, *bis*-phosphorylated structure of wild type *E. coli* lipid A (Fig 3.1a) is thought to be the ideal structure for maximal induction of TLR4/MD2 signaling, and removing acyl chains of lipid A decreases TLR4/MD2 activation[20,21]. In fact, remodeling lipid A after its synthesis affords many Gram-negative pathogens evasion of host TLR4/MD2 detection[2]. The length of lipid A acyl chains are generally speculated as important for proper insertion into MD2, but direct comparison between strains of the same background with altered acyl chain length has been limited, and data respecting altered secondary acyl chains is completely lacking.

Previous studies have initiated understanding of this question. Inactivating the cold-shock secondary palmitoleate (C16:1)-transferase, LpxP, in cold conditions causes a 10-fold decrease in minimum inhibitory concentration (MIC) to rifampicin and vancomycin, but not to other antibiotics tested[22]. However, this fatty acyl chain is unsaturated and likely alters various membrane properties to adapt to cold environments, such as membrane fluidity[23]. Other studies have altered the lengths of various acyl chains of lipid A and observed slight TLR4/MD2 activation phenotypes, but these studies have either used synthetic mimetics that vary structurally from lipid A and do not include

longer acyl chains[24], or lipid A samples that differ in multiple aspects of lipid A structure, making direct conclusions problematic. Some of these studies have also used only murine cell lines, but human and murine TLR4 differ greatly in their lipid A substrate specificity[2520,26]. One study did compare *B. pertussis* lipid A from isogenic strains altered only in the length of a single primary acyl chain and found increasing the length from C10 or C12 to a C14 caused a modest increase in human TLR4/MD2 activity[27].

Lipid A biosynthesis is tightly-regulated and well-conserved. In attempt to elucidate the benefits the particular lengths of secondary acyl chains *E. coli* lipid A, we have generated two sets of strains that range from 12-16 carbons in length at either the C2′- or C3′-position secondary acyl chains (Fig. 3.1b,c, 1.2)). Additionally, we have generated a strain in which the secondary acyl chain lengths are reversed compared to wild type (Fig 3.1d). Overall, stronger phenotypes were observed in membrane permeability than in TLR4/MD2 activation, indicating that the evolution of the acyl chain specificity of lipid A acyltransferases might have a stronger selective pressure for maintaining barrier integrity, while other lipid A modifications are sufficient to aid in evasion of the host immune response.

3.2 RESULTS

3.2.1 Altering the lengths of the secondary acyl chains on *E. coli* lipid A.

In order to generate two sets of strains that varied in length at either the 2′- or 3′-position secondary acyl chain, we replaced the genes encoding the *E. coli* acyltransferase enzymes LpxM and LpxL with heterologously expressed homologs (Fig. 1.2). The *lpxM* gene is nonessential, but *lpxL* mutants can only survive very slow growth on minimal media at low temperatures without compensatory expression of another copy of *lpxL* or one of its homologs[5,8].

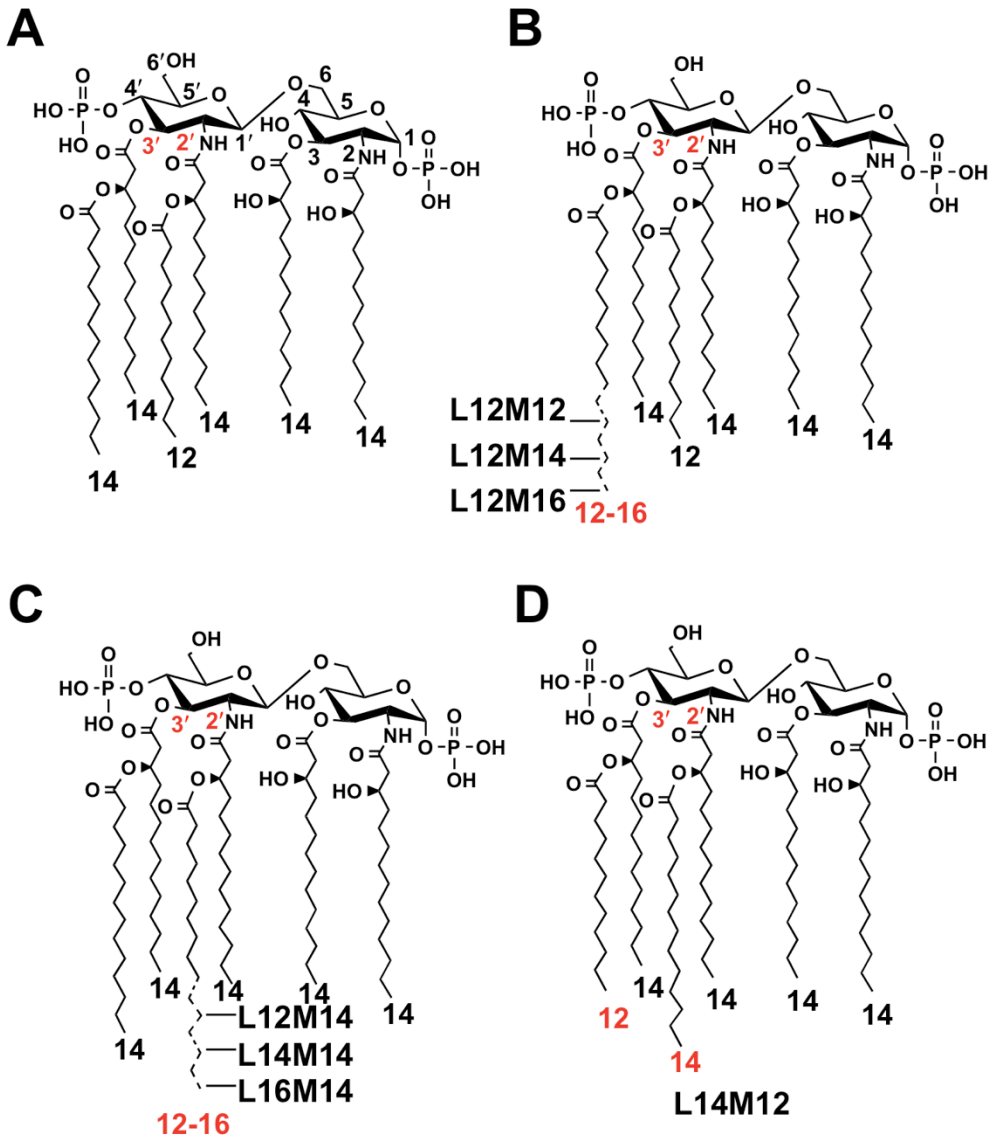


Figure 3.1 Lipid A from *E. coli* with varying lengths of secondary acyl chains.

(a) The structure of wild type *E. coli* lipid A. (b) Lipid A from engineered strains with varying lengths (C12-C16) of the 3′-position secondary acyl chain transferred onto the molecule by LpxM. (c) Lipid A from engineered strains with varying lengths (C12-C16) of the 2′-secondary acyl chain transferred onto the molecule by LpxL. (d) Structure of engineered lipid A with the lengths of the 3′- and 2′-position secondary acyl chains reversed compared to wild type.

First, a clean template strain was generated from the W3110 parent by deleting the genes encoding the lipid A modification enzymes EptA and LpxT, which transfer a phosphoethanolamine and a phosphate group onto the 1-position phosphate of lipid A (Fig. 1.1d, 2.2a)[17,28,29]. This strain expresses the native *E. coli lpxL* and *lpxM* acyltransferase genes and produces a homogeneous lipid A profile with a C12 secondary acyl chain at the 2′-position and a C14 secondary acyl chain at the 3′-position, and is thus referred to as L12M14 in this work (Fig. 3.1).

Further genetic manipulation of the L12M14 strain allowed alteration of the length of the secondary acyl chain added by LpxM at the 3′-position of lipid A. LpxM was deleted and recombinant *lpxM* genes could be expressed from the IPTG inducible plasmid, pQLinkN. Lipid A from the organisms *Yersinia pestis* and *Campylobacter jejuni* contain acyl chains at this position with lengths of C12 and C16, respectively[30,31]. In attempt to generate a strain with a laurate (C12) at this position, the *lpxM* homolog of *Yersinia pestis* (YPO2063) was expressed to generate the strain referred to as L12M12. Likewise, the gene responsible for acylation at the 3′-secondary position in *C. jejuni*, known as *lpxJ* (Cjj81176_0482) in this organism due to sequence divergence[31], was expressed from the plasmid to transfer a palmitoyl (C16) acyl chain onto lipid A and yield the strain L12M16.

To evaluate the lipid A in strains which differ only in the length of the C2′-position secondary acyl chain transferred by LpxL, the strains L14M14 and L16M14 were generated. Because LpxL is essential in rich broth at 37° C, *lpxL* homologs were heterologously expressed introduced into the L12M14 strain before deletion of the *E. coli lpxL* gene. *Vibrio cholerae* lipid A contains a C14 at the C2′-position and thus the *lpxL* gene (Vc0213) was chosen for expression to generate the L14M14 *E. coli* strain[32]. Increased expression of the *E. coli* LpxM was necessary in the L14M14 strain for

complete acylation, so the plasmid pACYC expressing $LpxM_{EC}$ was also introduced. To generate strains with longer secondary acyl chains at the C2′-position, *lpxL* equivalents from *C. jejuni*[33] and *Helicobacter pylori*[34] were expressed in strain L12M14 and the *E. coli lpxL* gene was deleted. *C. jejuni* and *H. pylori* lipid A contain a palmitoyl (C16) and stearoyl (C18) chain at this site, respectively, but when expressed in *E. coli*, we found that the *H. pylori* enzyme transferred primarily a C16 chain onto lipid A. We expect this is largely due to the shorter-chain pool of acyl-acyl carrier protein (acyl-ACP) donor substrate present in *E. coli* compared to *H. pylori*. Although *H. pylori lpxL* activity has previously been observed in *E. coli*, the length of acyl chain was not investigated[34]. Because the *H. pylori* LpxL enzyme was more efficient in *E. coli* than the *C. jejuni* LpxL, the strain containing the *H. pylori* LpxL was used for all further experiments and is designated L16M14. The expected lengths of chains and host organisms of these enzymes are summarized in the strain table (Table 5.2). Strains were tested for growth and all strains reached similar maximum yields to the wild type parent, W3110 and the template strain with wild type lipid A, L12M14. However, strain L14M14 had an increased lag phase. This could be due to the additional presence of the pACYC plasmid in this strain (Fig. 3.2).

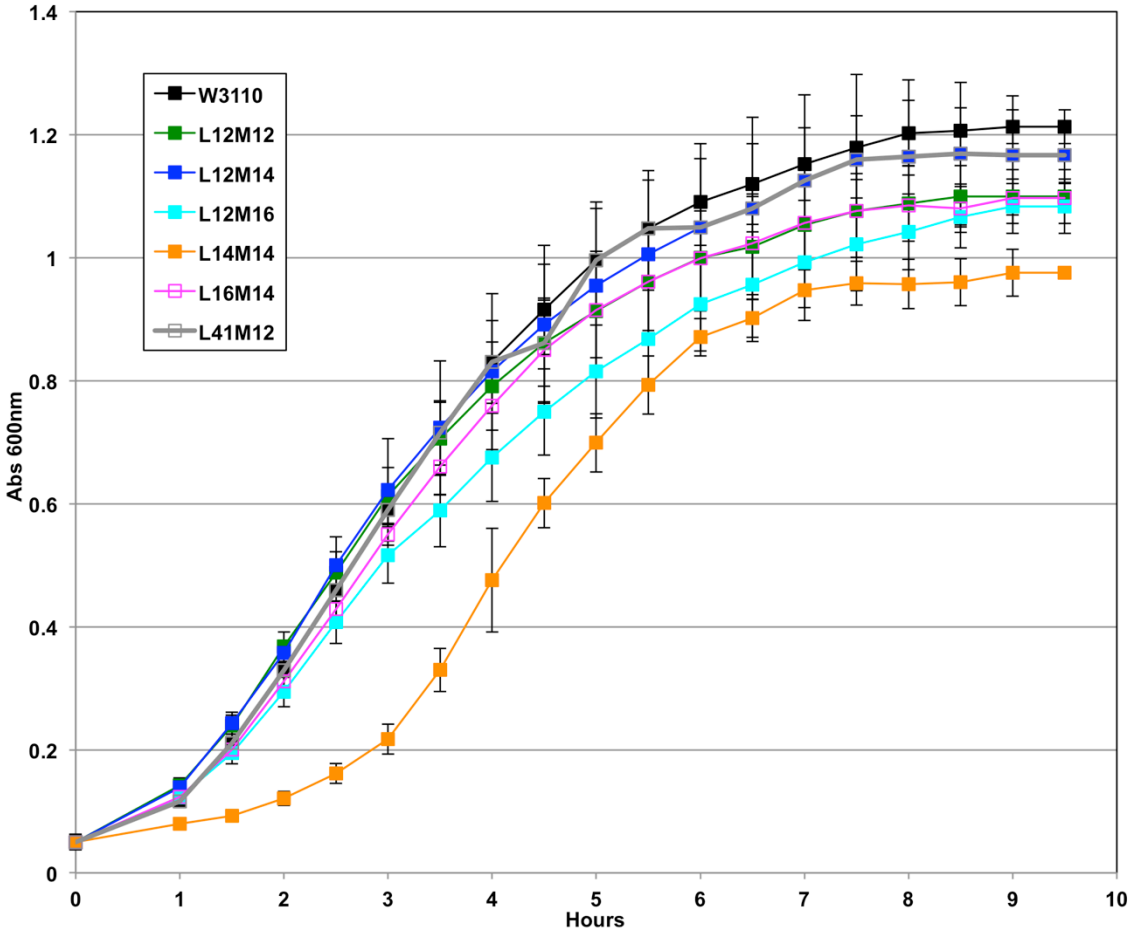


Figure 3.2 Growth curve of strains engineered for altered secondary acyl chain length of lipid A.

All strains were grown in LB at 37° C in the appropriate antibiotics and the OD_{600} was measured every 30 minutes. Most strains grew indistinguishable to wild type. Strain L14M14 displays an extended lag time and slightly lower maximal growth.

3.2.2 Analysis of lipid A profiles of acyl-engineered strains.

Consistent production of homogeneous lipid A containing altered secondary acyl chain length was confirmed by thin layer chromatography (TLC) and MALDI-TOF mass spectrometry (MS) for each strain. For TLC analysis, bacteria were grown in the presence of radioactive phosphate ($^{32}P_i$) and the lipid A was purified, separated, and visualized to verify successful incorporation of both secondary acyl chains onto the lipid A. This method is highly quantitative and illustrates the homogeneity of each hexa-acylated, *bis*-phosphorylated lipid A profile (Fig 3.3). Not surprisingly, the incremental lengthening of the acyl chains does not drastically alter the migration of lipid A on the plate.

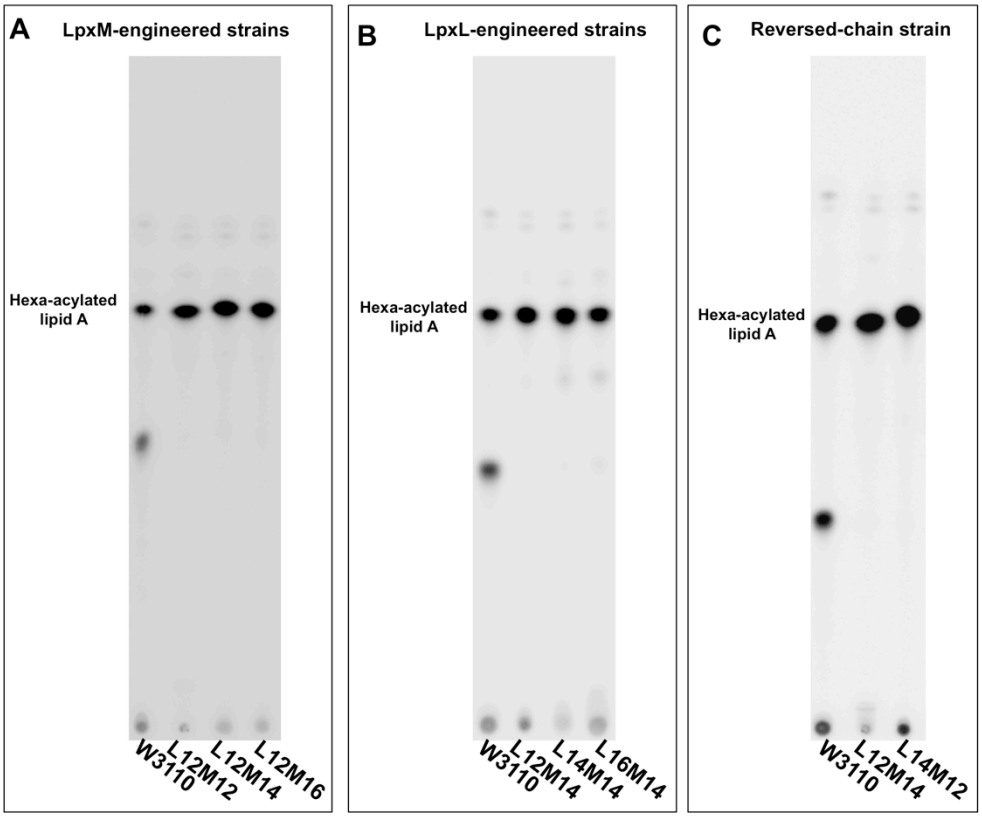


Figure 3.3 Thin layer chromatography (TLC) of $^{32}P_i$-labeled lipid A from acyl-engineered strains.

All strains were grown in the presence of $^{32}P_i$ and purified lipid A samples were separated by TLC. Lipid A from the wild type parent strain, W3110 is used as a migration control for hexa-acylated, *bis*-phosphorylated lipid A. Acyl chain mutants are also each compared to the template strain, L12M14, which is lacking lipid A modification enzyme LpxT, responsible for the slower migrating, phosphorylated lipid A species present in W3110 but absent in acyl-engineered strains. All strains, whether acyl chain length was altered at the 3′- (a) or 2′-position (b) secondary acyl chain, or both (c) demonstrated a homogeneous lipid A profile of hexa-acylated lipid A. Altering acyl chain length did not seem to affect migration of lipid A on the plate.

In order to identify the length of acyl chains in each strain and confirm the relative purity of each strain, mass spectrometry was performed. Analysis of strain L12M12 (Fig. 3.4a) results in a major peak with an m/z of 1769.7, which is consistent with lipid A containing a C12 at the secondary 3′-position. The predominant peak in strain L12M14 (Fig. 3.4b) at 1797.2 m/z is consistent with wild type lipid A chain length, and illustrates the expected mass increase of 27 m/z according to the addition of a single acyl chain that is two carbons longer than in lipid A from the L12M12 strain. Similarly, in the L12M16 strain, a major peak at m/z of 1824.2 is observed (Fig. 3.4c).

MS analysis of the strains with a range of lengths at the 2′-position secondary acyl chain also resulted in peaks consistent with expected masses. Fig 3.4d and 3.4e indicate major peaks at m/z 1824.1 and 1853.1 for the L14M14 and L16M14 strains, respectively. These masses correspond to lipid A molecules with the expected length of acyl chains and confirm the transfer of acyl chains by the heterologously expressed enzymes.

Analysis of strain L14M12 resulted in spectra quite similar to the template strain L12M14, as expected. The *V. cholerae* LpxL and *Y. pestis* LpxM were found to be quite specific in adding a C14 or C12, respectively, and we expect that these enzymes maintain their site-specificity in the L12M14 chain.

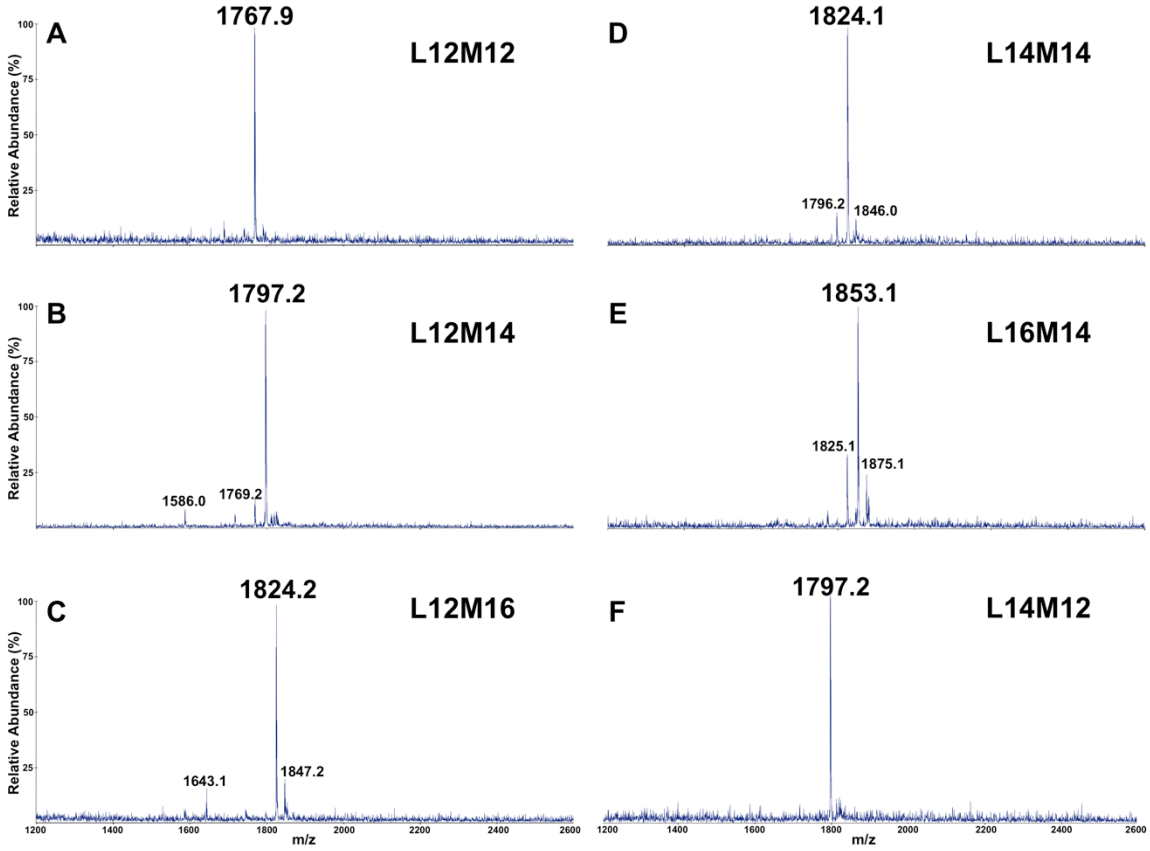


Figure 3.4 MS analysis of acyl-engineered lipid A from *E. coli*.

Lipid was purified from strains with engineered secondary acyl chain length and analyzed by negative ion MALDI/TOF mass spectrometry. (a-c) MS analysis of lipid A from strains varying in length at the 3′-position secondary acyl chain. (d and e) MS analysis of lipid A isolated from strains varying in length at the 2′-position secondary acyl chain. (f) MS analysis of strain L14M12 with the lengths of secondary acyl chains reversed compared to wild type lipid A.

3.2.3 TLR4 activation by whole cells and purified LPS from strains varying in secondary acyl chain length on lipid A.

It is well established that changes to the number and the arrangement of the acyl chains on lipid A have drastic effects on activation of the mammalian host receptor complex, TLR4/MD2 and the subsequent induction of cytokine signaling. The degree that lipid A fits snugly into the pocket of MD2 has a great effect on TLR4 activation. TLR4 antagonists with only four acyl chains, eritoran and lipid IV_A, fit deeper into the MD-2 pocket than hexa-acylated species, which limits the interactions between the lipid A substrate molecule and the two TLR4 dimers[35]. It is conceivable that lengthening or shortening the acyl chains of lipid A could have a similar effect, but the contribution of the length of acyl chains to this lipid A-MD2/TLR4 interaction has not been systematically studied.

To investigate the effect of altering the secondary acyl chains of lipid A on TLR4 activation, whole bacterial cells from each engineered strain were incubated with a human embryonic kidney (HEK) reporter cell line that secretes alkaline phosphatase upon TLR4 activation and signaling. Short chain lipid A could potentially fit deeper into the pocket of MD2, which could help or hinder association between the TLR4/MD2/lipid A homodimer. Similarly, long chain lipid A could impede sufficient insertion into the MD2 pocket and partially disrupt the complex. However, we were surprised to find that TLR4 assays resulted in quite similar activation curves for each strain (Fig. 3.5). At low cell densities (10^2-10^5), longer secondary acyl chains at the 2′-position (Strains L14M14 and L16M14) resulted in a modest decrease in TLR4 stimulation, and a shorter secondary acyl chain at the 3′-position (strain L12M12) slightly increased activation. However, at high cell densities, all the strains are as agonistic as wild type lipid A. Rearranging the

acyl chains in the L12M14 and the L14M12 strains also had little effect on TLR4 activation.

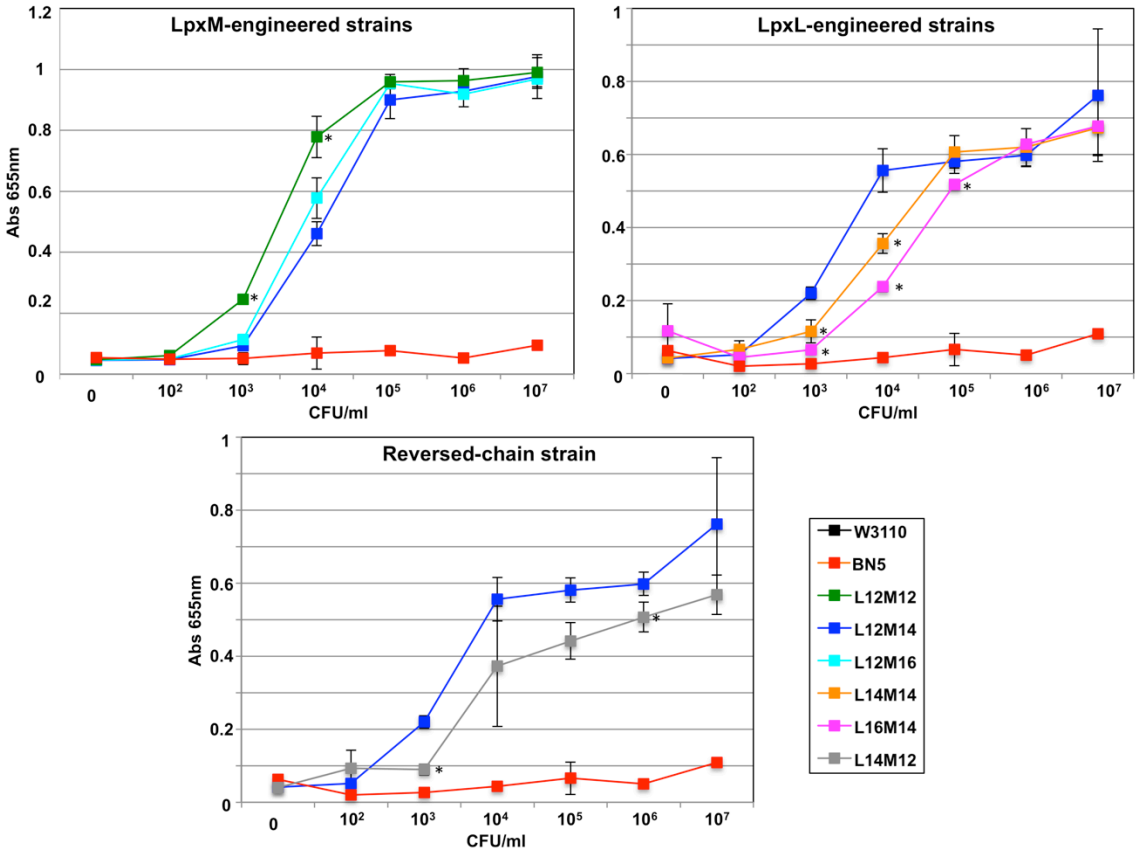


Figure 3.5 TLR4/MD2 activation by whole bacteria cells with varying lengths of secondary acyl chains.

Increasing cell numbers were incubated with HEK293 cells expressing human TLR4 and its cofactors MD2 and CD14 and a reporter alkaline phosphatase system for detection of activation of TLR4 signaling. Non-stimulatory, penta-acylated strain BN5 was used as a negative control. Top panel, strains varying in acyl chain length at the 3′-position secondary acyl chain are compared to each other and BN5. Bottom panel, strains varying in acyl chain length at the 2′-position secondary acyl chain as well as the strain with reversed length of secondary acyl chains are compared to each other and BN5. A p-value of <0.05 at any given concentration relative to the L12M14 strain is indicated by an asterisk.

A similar phenomenon occurred when purified LPS from each strain was incubated with the HEK-TLR4 reporter cells. At low LPS concentrations (0.01-1ng/ml), the presence of the C16 acyl chain caused a slight decrease in TLR4 activation, with a stronger effect when this chain was in the 2′-position. However, at high LPS concentrations (10-100 ng/ml shown, up to 10 ug/ml tested), each sample was indistinguishable (Fig. 3.6). When using LPS from the L12M14 and L14M12 strains in which the secondary acyl chain lengths are reversed, no difference in TLR4 activation was seen (Fig 3.6, bottom). Although modest effects on TLR4 activation were observed at low doses of cells and LPS, the overall effect of altering the length of secondary acyl chains of lipid A is quite minimal.

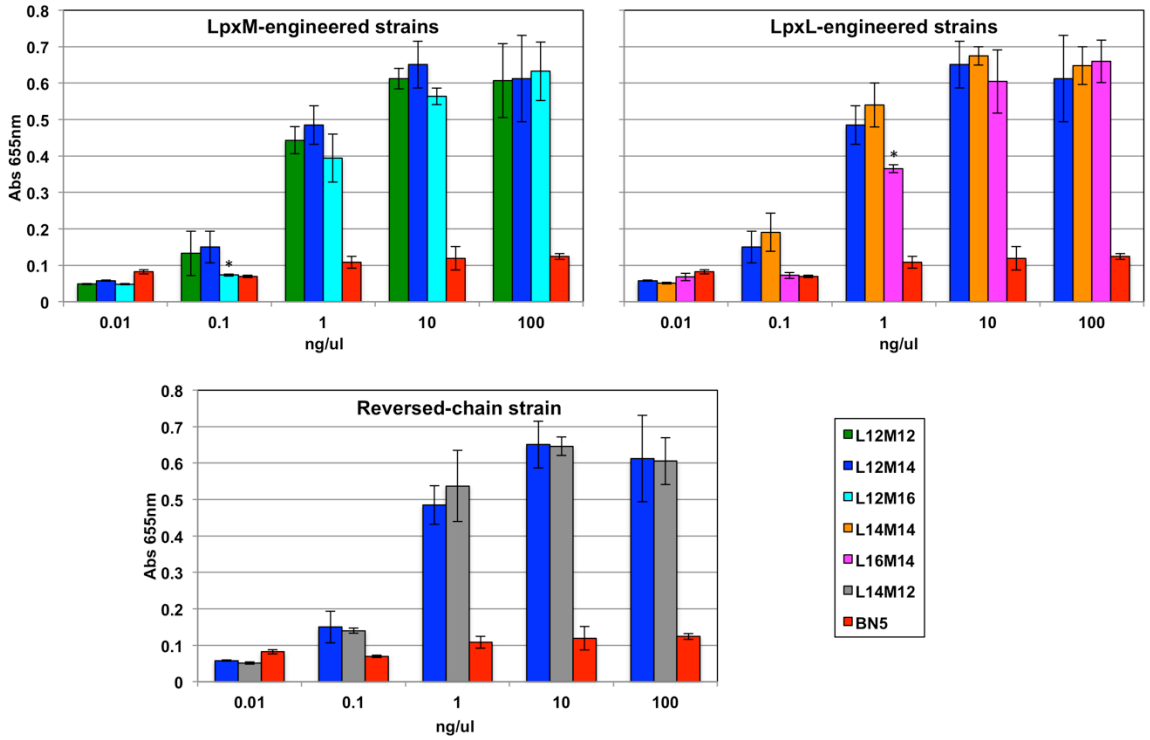


Figure 3.6 TLR4/MD2 activation by purified LPS from strains with varying lengths of secondary acyl chains.

Increasing concentrations of LPS were incubated with HEK293 cells expressing human TLR4 and its cofactors MD2 and CD14 and a reporter alkaline phosphatase system for detection of activation of TLR4 signaling. LPS from unstimulatory, penta-acylated strain BN5 was used as a negative control. Top panel, activation by LPS from strains varying in acyl chain length at the 3′-position secondary acyl chain are compared to each other and BN5. Middle panel, activation by LPS from strains varying in acyl chain length at the 2′-position secondary acyl chain are compared to each other and BN5. Bottom panel, activation by LPS from the strain with reversed arrangement of secondary acyl chains is compared to LPS from wild type secondary acyl chain length and BN5. A p-value of <0.05 at any given concentration relative to the L12M14 strain is indicated by an asterisk.

3.2.4 Analysis of the outer membrane permeability barrier in strains with acyl-engineered lipid A.

Gram-negative bacteria tightly control the lengths of each acyl chain incorporated into their lipid A. However, among species, variation exists in these length specificities. Since the effect on TLR4 signaling appears to be minimal, we investigated the membrane properties of the strains with varying length of secondary acyl chains and found that *E. coli* may have evolved specificity in lipid A acyl chain length that is crucial to maintain a robust permeability barrier.

To test the outer membrane permeability barrier, we grew each strain (at least three biological replicates done in duplicate) in LB and various membrane stressors and small molecules designed to probe for membrane permeability as follows: bile salts (Fig. 3.7), ethylenediaminetetraacetic acid (EDTA) (Fig. 3.8), sodium dodecyl sulfate (SDS) (Fig. 3.9), and vancomycin (Fig. 3.10).

As an enteric organism, *E. coli* is well-adapted to resist high concentrations of membrane perturbing agents found in the gastrointestinal tract like bile and similar compounds, such as SDS[36]. Gram-negative bacteria are also naturally resistant to some antimicrobials that swiftly eliminate Gram-positive pathogens, such as vancomycin[37]. EDTA, a metal chelator, disturbs the cationic bridges that stabilize charges between the negative phosphate groups of lipid A[38]. Various mechanisms contribute to survival in such environments, but resistance is largely due to the outer membrane's effectiveness at protruding such molecules.

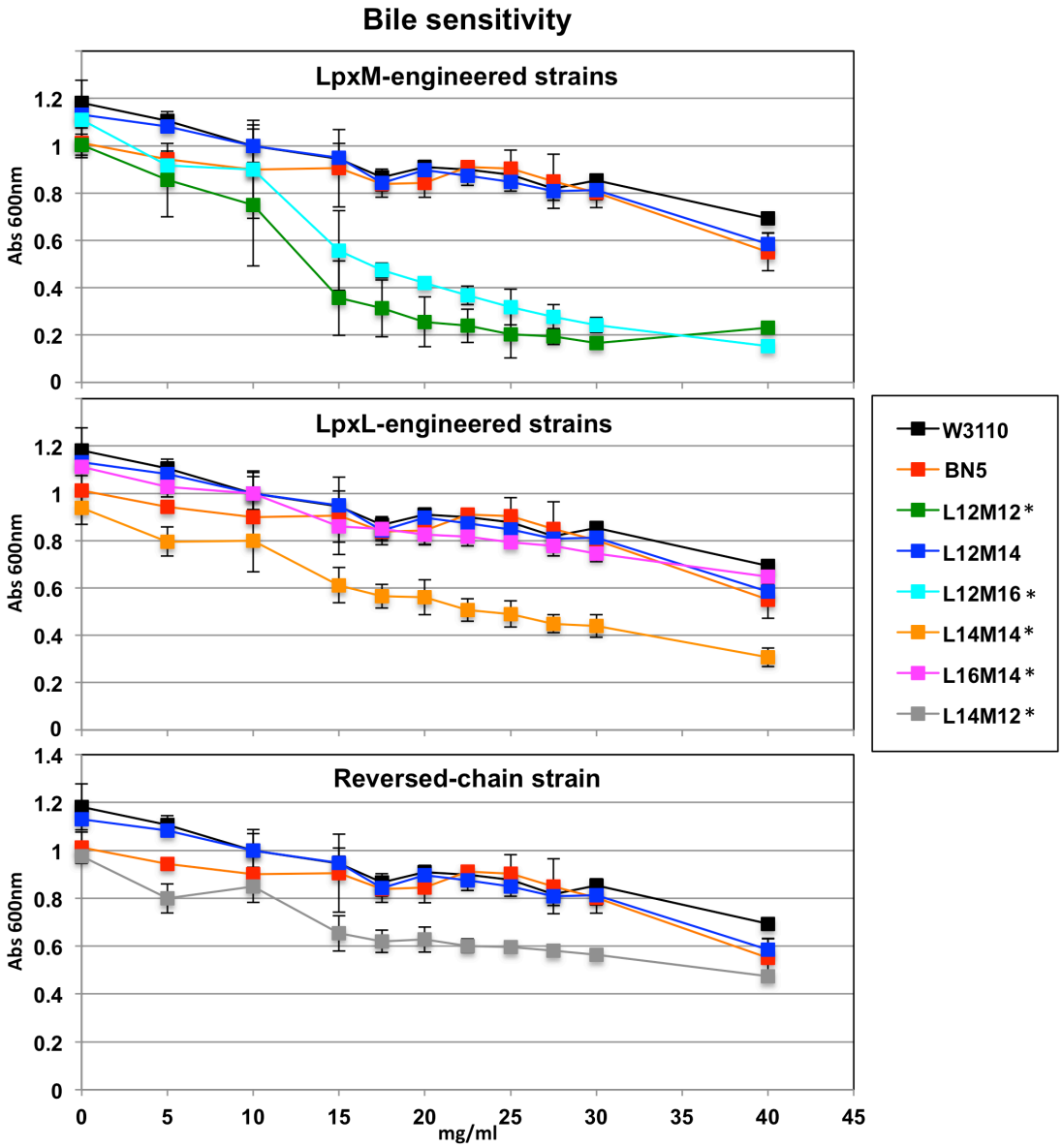


Figure 3.7 Growth of acyl-engineered strains in the presence of bile.

Growth of all strains including the W3110 parent and the penta-acylated LpxM mutant (BN5) was performed in LB in the presence of increasing concentrations of bile for 12 hrs and the OD_{600} was measured. The top panel represents growth of strains modified only at the 3′-position secondary acyl chain transferred onto lipid A by LpxM. The middle panel shows results for the 2′-position secondary acyl chain transferred onto lipid A by LpxL. The bottom panel represents growth of the strain with both secondary acyl chains reversed compared to wild type. A p-value of <0.05 relative to the L12M14 strain at 20 mg/ml is indicated by an asterisk in the legend.

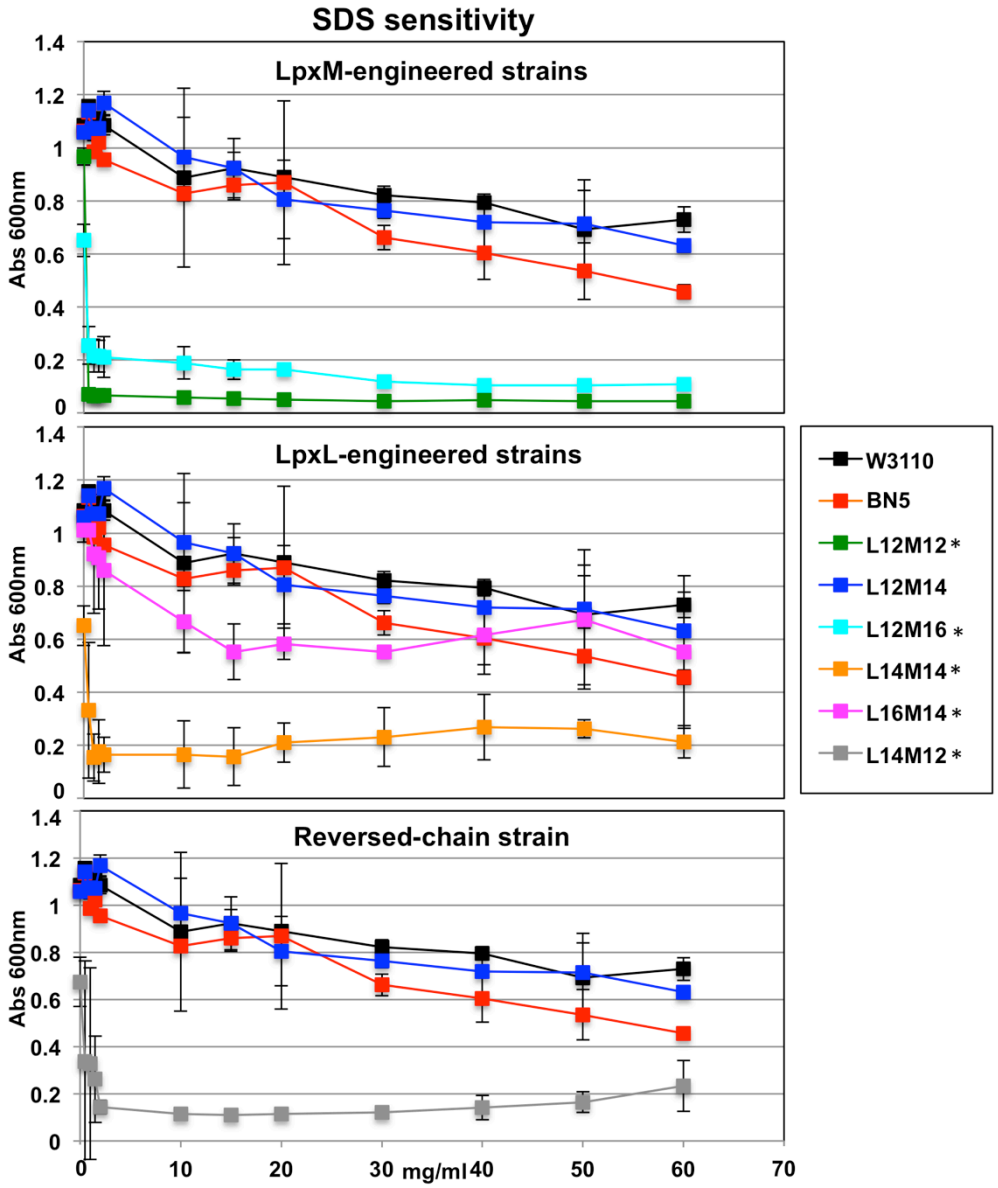


Figure 3.8 Growth of acyl-engineered strains in the presence of SDS.

Growth of all strains including the W3110 parent and the penta-acylated *lpxM* mutant (BN5) was performed in LB in the presence of increasing concentrations of SDS for 12 hrs and the OD_{600} was measured. The top panel represents growth of strains modified only at the 3′-position secondary acyl chain transferred onto lipid A by LpxM. The middle panel shows results for the 2′-position secondary acyl chain transferred onto lipid A by LpxL. The bottom panel represents growth of the strain with both secondary acyl chains reversed compared to wild type. A p-value of <0.05 relative to the L12M14 strain at 30 mg/ml is indicated by an asterisk in the legend.

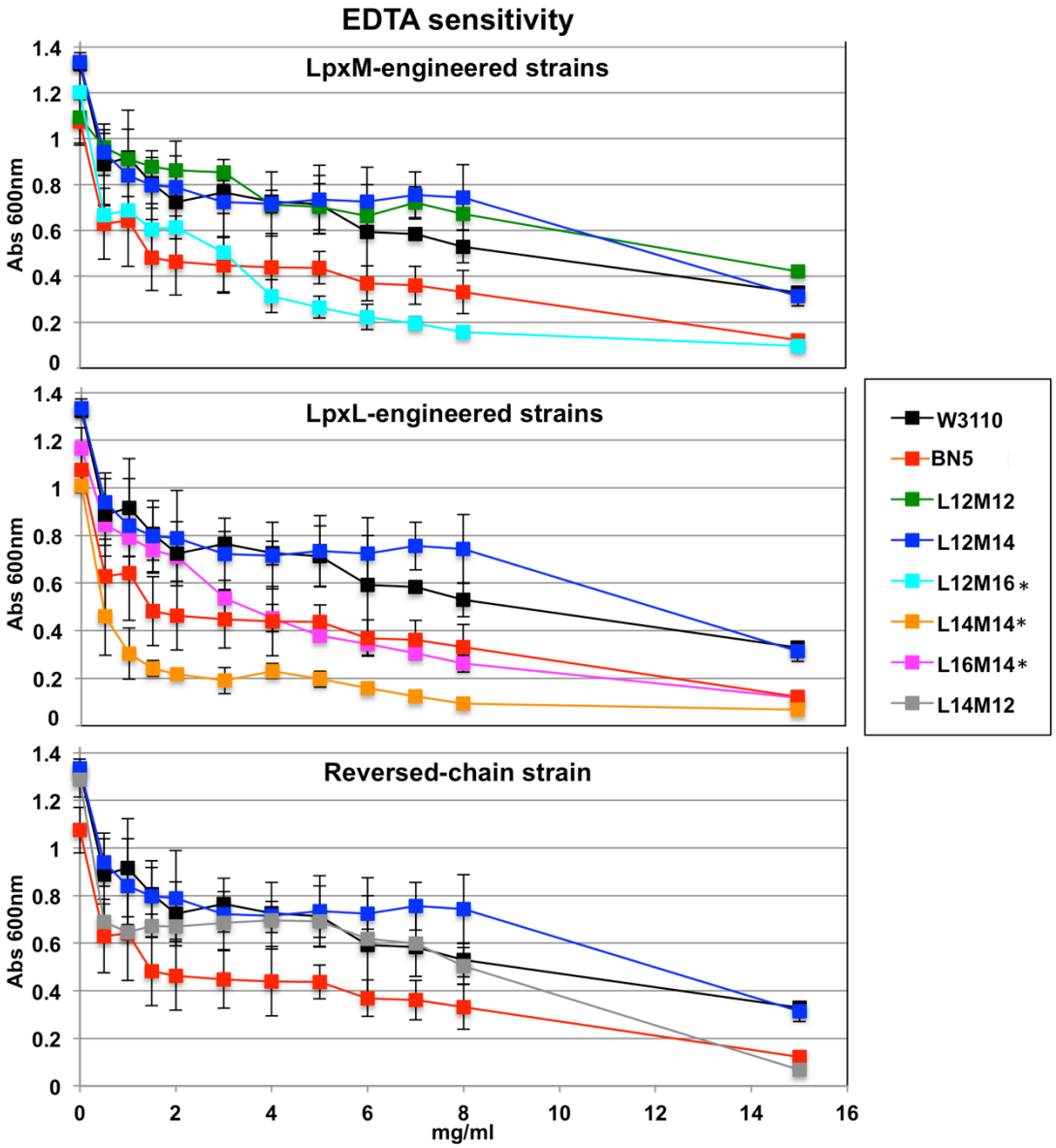


Figure 3.9 Growth of acyl-engineered strains in the presence of EDTA.

Growth of all strains including the W3110 parent and the penta-acylated LpxM mutant (BN5) was performed in LB in the presence of increasing concentrations of EDTA for 12 hrs and the OD_{600} was measured. The top panel represents growth of strains modified only at the 3′-position secondary acyl chain transferred onto lipid A by LpxM. The middle panel shows results for the 2′-position secondary acyl chain transferred onto lipid A by LpxL. The bottom panel represents growth of the strain with both secondary acyl chains reversed compared to wild type. A p-value of <0.05 relative to the L12M14 strain at 6 mg/ml is indicated by an asterisk in the legend.

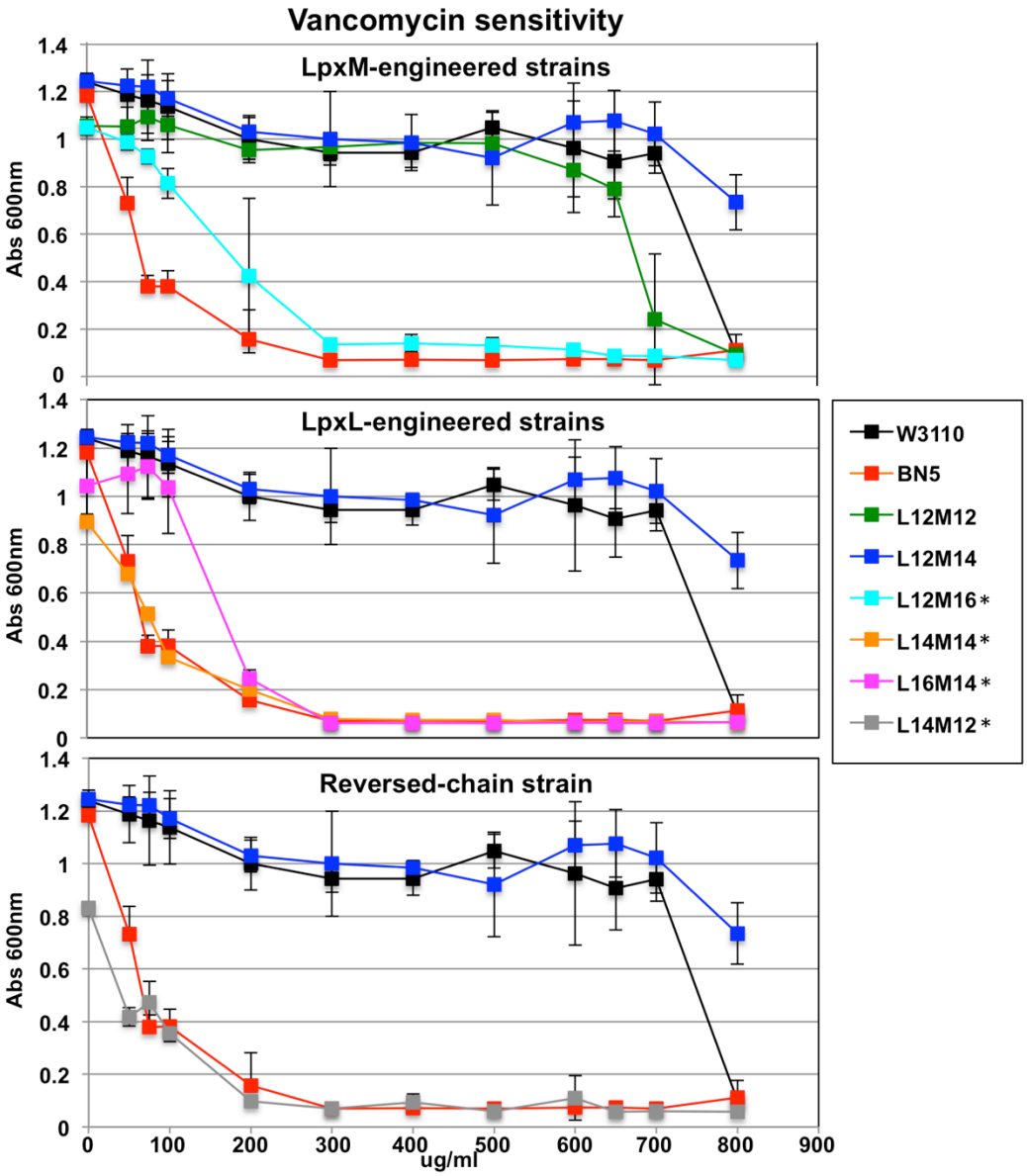


Figure 3.10 Growth of acyl-engineered strains in the presence of vancomycin.

Growth of all strains including the W3110 parent and the penta-acylated LpxM mutant (BN5) was performed in LB in the presence of increasing concentrations of vancomycin for 12 hrs and the OD_{600} was measured. The top panel represents growth of strains modified only at the 3′-position secondary acyl chain transferred onto lipid A by LpxM. The middle panel shows results for the 2′-position secondary acyl chain transferred onto lipid A by LpxL. The bottom panel represents growth of the strain with both secondary acyl chains reversed compared to wild type. A p-value of <0.05 relative to the L12M14 strain at 400 ug/ml is indicated by an asterisk in the legend.

In all conditions tested, strain L12M14 with wild type secondary acyl chain length grew similarly to the parent strain, W3110. Strain L12M12, with a shorter secondary acyl chain at the LpxM position grew like wild type in vancomycin and EDTA, but was severely compromised in bile and SDS. When that same secondary acyl chain was lengthened to a C16 in strain L12M16, low intermediate growth in SDS and vancomycin occurred, but drastic sensitivity to EDTA and bile were observed.

Altering the acyl chain at the LpxL position also affected growth in the various compounds. Strain L14M14 was sensitive to all compounds tested but strain L16M14 grew at high intermediate levels in bile, SDS, and EDTA, although it was sensitive to vancomycin.

When strain L14M12 with reversed secondary acyl chain length was compared to the L12M14 strain with wild type acyl chains, high sensitivity to vancomycin and SDS was observed, but growth in EDTA and bile was observed to be similar to the wild type strain.

3.3 DISCUSSION

Gram-negative bacteria frequently modify the lipid A covering the cell surface to adapt to the diverse environments they encounter. However, acyl chain length of lipid A remains quite consistent amidst the addition or removal of various types of chemical groups from the molecule. In some cases, like removal of an entire acyl chain, these diverse modifications aid the bacteria in evasion of the innate immune response by decreasing stimulation of the TLR4/MD2 host LPS receptor[394]. Other modifications, such as masking the charge of the phosphate groups of lipid A with an amine-containing moiety, primarily benefit the bacterium by limiting association of antimicrobial

molecules like CAMPS to the cell surface[17]. The work described herein investigated how the specific lengths of the secondary acyl chains of *E. coli* lipid A affect initiating host immune detection by TLR4/MD2 and resistance to compounds that perturb the outer membrane permeability barrier.

To study these aspects of lipid A in a controlled system with no other variations in lipid A structure, we have generated two sets of strains with altered length at the 2′-position secondary acyl chain incorporated by the acyltransferase LpxL (Fig. 3.1b) or the 3′-position secondary acyl chain transferred onto the molecule by the acyltransferase LpxM (Fig. 3.1c). Additionally, we generated a strain in which the lengths of the secondary acyl chains are reversed in the two positions, relative to wild type (Fig 3.1d). In spite of the general idea in the field that acyl chain length plays a significant role in TLR4 activation, varying the secondary acyl chains of lipid A from C12 to C16 had little effect on activity. When the HEK reporter cell line was incubated with low bacterial cell numbers or low concentrations of LPS from each strain, a shorter acyl chain (C12) at the LpxM position slightly increased activation, while longer acyl chains (C14 and C16) at the LpxL position slightly decreased it. However, at high concentrations of both cells and purified LPS, the lipid A with any of the lengths of secondary acyl chains was maximally stimulatory.

Upon investigating the growth of each acyl-engineered strain in the presence of compounds that perturb the membrane or are normally excluded from the cell in Gram-negative bacteria, it was apparent that secondary acyl chain length was crucial for proper integrity of the cell surface. Altering the length of either the 2′- and 3′-position secondary acyl chains affected bacterial fitness in bile, EDTA, SDS, and vancomycin. Apparently, *E. coli* has evolved precise acyltransferase enzymes in order to establish a perfect harmony of acyl chain length it its membranes. While acyl chain length is tightly

regulated and consistent within a species, it can differ greatly between organisms. It is likely that each have an intricate balance between the barrier qualities of the lipids and proteins that comprise their membranes.

Chapter 4: Conclusion[3]

4.1 Importance for the continued study of lipid A

4.1.1 Lipid A vaccine adjuvants and therapeutics.

A wide variety of lipid A modifications equip Gram-negative bacteria for survival in their respective niches in the host and other environments. The lipid A molecule as well as the many biosynthetic and modification enzymes that have evolved provide a tool kit for potential immune system modulation in therapeutics. In this work, we have studied the range of lipid A modifications that can be made to acyl chain number and arrangement in combination with the loss of one or both phosphate groups. Our hope is that some of these combinatorial strains will prove useful for vaccine development. We have also generated strains modified in acyl chain length, which apparently do not modulate the immune system, but do offer insight into bacterial fitness. All of these strains provide a unique resource for future studies involving endotoxin, the outer membrane, and Gram-negative bacteria.

4.1.2 Effects on other cellular processes.

A better understanding of how lipid A biosynthetic and modification enzymes affect lipid A, the outer membrane, and pathogenesis will also greatly contribute to efforts for the control of infectious disease. For example, functions of lipid A are highlighted by the interplay of lipid A modifications with a diverse set of cellular processes and both of these facets of lipid A biology emphasize the importance for a continued effort to understand the regulation, benefits and host response to lipid A in its numerous modified forms. Recent studies have revealed links between lipid A

[3] Large portions of this chapter have been previously published. Needham, B. D. & Trent, M. S. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. *Nat. Rev. Microbiol.* **11,** 467–481 (2013).

modifications and diverse processes within the bacterial cell. These associations support the notion that lipid A modifications are crucial for bacterial pathogenesis and survival.

4.1.2.1 Multitarget lipid A modification enzymes

In *Campylobacter jejuni*, the enzyme EptC modifies both lipid A and a structural protein required for flagellar assembly. EptC is a homologue of *E.coli* EptA and both enzymes transfer a phosphoethanolamine residue to the 1′- and 4′-positions of lipid A (see Table 1), masking both phosphate groups and promoting CAMP resistance. Interestingly, in *C. jejuni* EptC also transfers an essential phosphoethanolamine to FlgG (a flagellar rod protein[135]) and EptC mutants lack wild type motility and produce fewer flagella, which is required for virulence. This promiscuous enzyme also modifies carbohydrates of the *C. jejuni* LOS core and glycosylated proteins[136].

4.1.2.2 Recycling of biosynthetic intermediates

During the assembly of bacterial polymers (for example, peptidoglycan, the capsule and LPS) a wide array of glycoconjugates must be trafficked across the inner membrane[137]. These glycan intermediates are assembled on a universal carrier lipid known as undecaprenyl-phosphate (C_{55}-P)[138]. The C_{55}-P transports the glycan precursors in a pyrophosphate linkage (C_{55}-PP-precursor) and when the glycan is removed, the lipid is released as a pyrophosphate and requires dephosphorylation for the carrier to be recycled[138]. Within the periplasm, LpxT transfers a secondary phosphate group onto lipid A at the 1-position (Fig. 4.1C) and uses the C_{55}-PP as a donor[105]. This finding represented a novel and unexpected link between the modification of lipid A and the recycling of C_{55}-PP. Furthermore, the connection between these systems revealed that C_{55}-PP could serve as a high energy phosphate donor for various periplasmic components, although these potential targets are largely unidentified.

4.1.2.3 Outer membrane protein activation

As a membrane component, LPS is more than just a platform for activities at the cell surface[139]. It also has a role in the activation of a class of proteases that are widespread among Gram-negative enteric bacteria, known as omptins. The activity of one member of this class (OmpT protease) has been evaluated in the presence of hexa-acylated and tri-acylated lipid A, and was found to be only active in the presence of hexa-acylated lipid A[140]. Interestingly, another omptin named plasminogen (Pla), which is central to *Y. pestis* pathogenesis, is more active in *Y. pestis* grown at 37° C and producing tetra-acylated lipid A, compared to cells grown at 20° C and producing hexa-acylated lipid A[141].

4.1.2.4 Outer membrane vesicles and toxin delivery

Modifications of lipid A also affect the delivery of proteins and toxins that are associated with the outer membrane and outer membrane vesicles (OMVs). In organisms such as *Porphyromonas gingivalis* and *P. aeruginosa*, negatively charged LPS is enriched in OMVs[142,143]. Furthermore, in *P. gingivalis* the lipid A found in OMVs is more deacylated compared to the lipid A that is present in whole cell membranes, suggesting that lipid A modification could facilitate vesicle formation or the sorting of outer membrane proteins that are packaged into vesicles[143]. In *P. aeruginosa*, OMV formation is stimulated by a small signalling molecule, *Pseudomona*s Quinolone Signal (PQS), which is packaged into vesicles and induces vesicle formation in *P. aeruginosa* cells as well as other bacteria[144]. The exact mechanism of OMV formation is not understood; however, PQS was found to interact with the 4′-phosphate group and acyl chains of lipid A, indicating that modification of lipid A could influence the efficiency of vesicle shedding[145].

The structure of lipid A and the Kdo residues of LPS also affect the delivery of toxins. For example, two similar toxins - cholera toxin (CT) produced by *V. cholerae* and heat-labile toxin (LT) produced by Enterotoxigenic *E. coli* (ETEC) - are both secreted, are structurally similar and bind the same host cell receptor. However, CT causes a more severe diarrheal disease[146]. Although the topic is controversial[147], the difference in toxin severity seems to be due to the partial sequestration of LT as it binds to Kdo-lipid A on the bacterial cell surface after secretion. By contrast the Kdo sugar attached to lipid A in *V. cholerae* is phosphorylated by the kinase KdkA (Table 1.1), and this modification inhibits CT association with the outer membrane, thereby allowing robust toxin secretion[148].

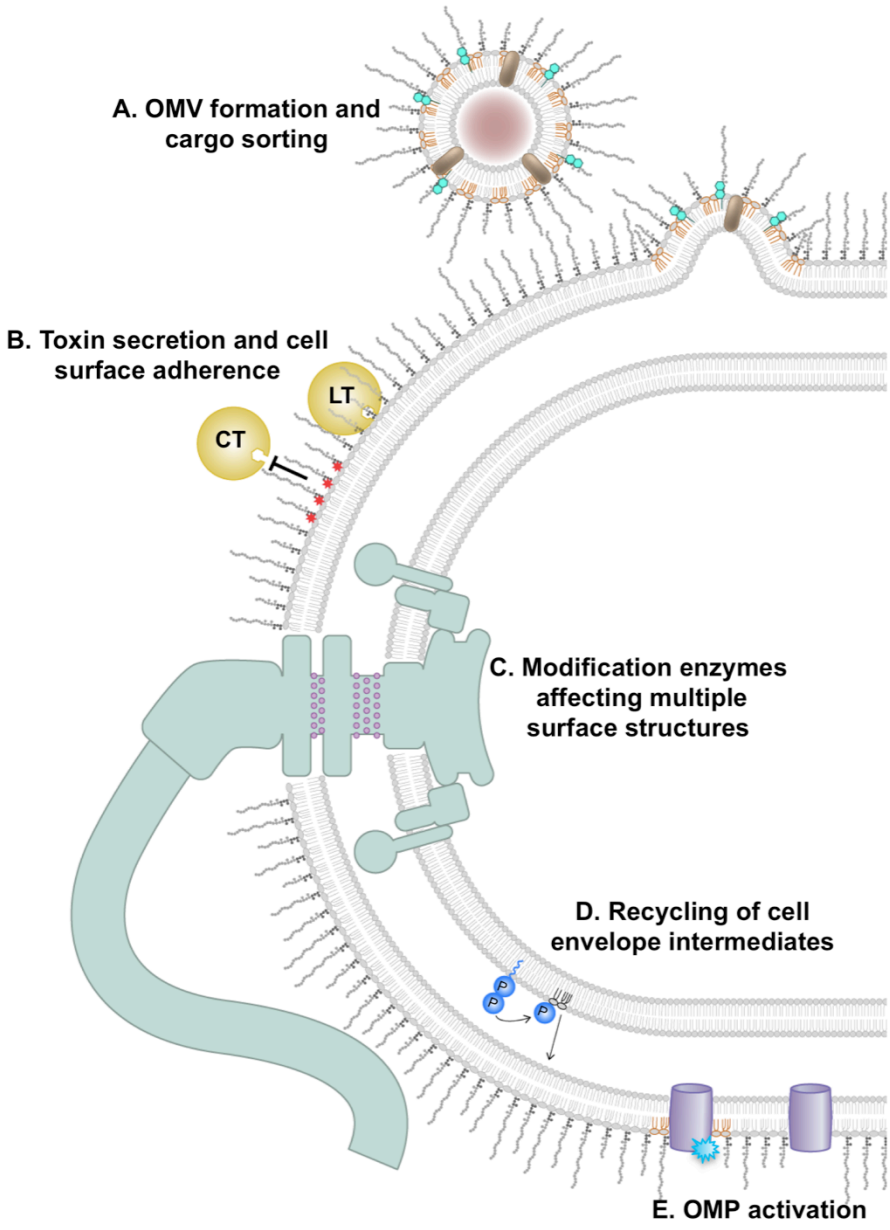


Figure 4.1 Networks between lipid A modifications and other cellular processes

Depicted here are examples of the variety of networks between lipid A modification enzymes and various other virulence factors and functions in the cell. Connections with outer membrane vesicle (OMV) formation, toxin secretion and delivery, motility, recycling of undecaprenyl pyrophosphate (C_{55}-PP), and activation of outer membrane proteins (OMP) have been identified.

4.2 FUTURE QUESTIONS TO BE ADDRESSED IN THE LIPID A FIELD

In spite of the continual progress studying the Gram-negative outer membrane and its components, many questions remain unanswered. The continued development of animal models is paramount to more closely mimic the host infection site. Considerable progress has been made in this regard by the recent generation of a transgenic mouse expressing the human TLR4 receptor[149] There is much to learn about how lipid A and its modifications affect the outcome of polymicrobial infections, with recent studies suggesting that LPS has an important role within such communities. For instance, purified LPS from intestinal commensal bacteria has been found to promote replication and transmission of viruses[150,151]. Pathogenesis of orally acquired poliovirus, which replicates in the intestine before spreading to cause a severe systemic infection, is supported by the intestinal microbiota and purified LPS[151]. Mouse mammary tumor virus (MMTV) manipulates the innate immune response by binding LPS and eliciting TLR4-dependent production of interleukin-10, a cytokine that is required for MMTV persistence[150]. Although these viruses bind LPS and this enhances infectivity *in vitro*, how lipid A modifications affect these phenomena is currently unknown**.**

Improved methods for studying lipid A modifications *in vivo* could also help elucidate any potential links between modified lipid A and diseases involving TLR4, such as inflammatory bowel disease, diabetes, rheumatoid arthritis and atherosclerosis[152]. Important insights into human TLR4 polymorphisms might now be possible using the humanized mouse model[149]; and it should also be possible to examine how different variants of TLR4 affect susceptibility to infection by organisms with modified lipid A. This could potentially lead to more personalized medical treatment through the development of a new range of modified TLR4 agonists that could serve as customized immunomodulatory agents to induce or repress particular cytokine responses.

It remains a mystery why lipid A is essential in virtually all Gram-negative organisms. Furthermore, why lipid A biosynthesis is well-conserved only to have complex modifications occur later, sometimes constitutively, is not understood. How the modification enzymes are distributed and organized within the membrane is also unclear. For example, it is not known whether lipid rafts exist in bacteria and if some or all of the modification enzymes are enriched at these sites. Lipid A molecules with certain modifications could also be specifically localized in the membrane. Such an organization could have a major influence on the activation of key outer membrane proteins or OMV formation and protein sorting.

With further study, inhibitors of modification enzymes could be developed for use as antibiotics or in combination with currently used antibiotics. Although immense progress has been made in our understanding of lipid A modification systems in Gram-negative bacteria and their importance for pathogenesis, there is much work yet to be done.

Chapter 5: Experimental Procedures

5.1 CONSTRUCTION OF MUTANT STRAINS

5.1.1 Mutants generated in Chapter 2.

Construction of mutant strains was performed by P1 *vir* phage transduction using Keio mutants, as previously described[105]. BN1 was generated by deletion of *pagP* from BN0, an *lpxT* and *eptA* double mutant[8,35]. Deletion of all three genes resulted in a strain producing >95% of the prototypical, hexa-acylated *bis*-phosphorylated lipid A species. To double the potential lipid A profiles that one set of enzymes could yield, BN2 was generated by removal of the Kan^R cassette and deletion of *lpxM* from BN1. Strains were confirmed by PCR, $^{32}P_i$ radiolabeling, and MS[153] (Table 5.1 for strains and plasmids).

5.2.2 Mutants generated in Chapter 3.

Gene deletions of *eptA, lpxT, and lpxM*[35,154] and subsequent removal of each antibiotic cassette[155] were performed by P1 *vir* phage transduction using Keio mutants, as previously described. Mutation of *lpxL* was performed using P1 *vir* phage transduction using the previously described mutant MKV15B[122]. LpxL mutation was only performed after introduction of a covering pQLinkN plasmid expressing an *lpxL* homolog (see Table 5.2 for plasmids). The plasmids encoding *lpxM* homologs were transformed into strain BN5. To generate strain L14M12, the pQ-$L_{VC}M_{YP}$ plasmid was introduced into the BN5 strain followed by deletion of *lpxL* using MKV15B phage. Strains were confirmed by PCR, $32P_i$ radiolabeling, and MS.

Table 5.1 Bacterial strains and plasmids used for chapter 2.

Strain or plasmid	Genotype or description	Source or reference
Strains		
W3110	Wild type, F⁻ I⁻ rph-1 INV(*rrnD, rrnE*)1 *rph-1*	*E. coli* Genetic Stock center (Yale)
MLK1067	W3110 *lpxM*::Ω*cam*	156
CMR300	W3110 (*kdtA*::*kan*) pWMsbA	157
BN0	W3110 Δ*eptA, ΔlpxT*	This work
BN1	BN0 Δ*pagP*	This work
BN2	BN1 Δ*lpxM::kan*	This work
Plasmids		
pQLinkN	Vector containing a tac promotor, Amp^r	106
pE	pQLinkN containing *lpxE*	This work
pF	pQLinkN containing *lpxF*	This work
pL	pQLinkN containing *pagL*	This work
pO	pQLinkN containing *lpxO*	This work
pP	pQLinkN containing *pagP*	This work
pR	pQLinkN containing *lpxR*	This work
pEF	pQLinkN containing *lpxE, lpxF*	This work
pEL	pQLinkN containing *lpxE, pagL*	This work
pEO	pQLinkN containing *lpxE, lpxO*	This work
pEP	pQLinkN containing *lpxE, pagP*	This work
pER	pQLinkN containing *lpxE, lpxR*	This work
pFL	pQLinkN containing *lpxF, pagL*	This work
pFP	pQLinkN containing *lpxF, pagP*	This work
pFR	pQLinkN containing *lpxF. lpxR*	This work
pLO	pQLinkN containing *pagL, lpxO*	This work
pLP	pQLinkN containing *pagL, pagP*	This work
pLR	pQLinkN containing *pagL, lpxR*	This work
pOP	pQLinkN containing *lpxO, pagP*	This work
pOR	pQLinkN containing *lpxO, lpxR*	This work
pPR	pQLinkN containing *pagP, lpxR*	This work
pEFL	pQLinkN containing *lpxE, lpxF, pagL*	This work
pEFP	pQLinkN containing *lpxE, lpxF, pagP*	This work
pEFR	pQLinkN containing *lpxE, lpxF, lpxR*	This work
pELO	pQLinkN containing *lpxE, pagL, lpxO*	This work
pELP	pQLinkN containing *lpxE, pagL, pagP*	This work
pELR	pQLinkN containing *lpxE, pagL, lpxR*	This work
pEOP	pQLinkN containing *lpxE, lpxO, pagP*	This work
pEPR	pQLinkN containing *lpxE, pagP, lpxR*	This work
pFLP	pQLinkN containing *lpxR, pagL, pagP*	This work
pFLR	pQLinkN containing *lpxF, pagL, lpxR*	This work
pFPR	pQLinkN containing *lpxF, pagP, lpxR*	This work
pLOP	pQLinkN containing *pagL, lpxO, pagP*	This work
pLOR	pQLinkN containing *pagL, lpxO, lpxR*	This work
pLPR	pQLinkN containing *pagL, pagP, lpxR*	This work

Table 5.1 continued.

pOPR	pQLinkN containing *lpxO, pagP, lpxR*	This work
pEFLP	pQLinkN containing *lpxE, lpxF, pagL, pagP*	This work
pEFLR	pQLinkN containing *lpxE. lpxF, pagL, lpxR*	This work
pEFPR	pQLinkN containing *lpxE, lpxF, pagP, lpxR*	This work
pELOP	pQLinkN containing *lpxE. pagL, lpxO, pagP*	This work
pELOR	pQLinkN containing *lpxE. pagL, lpxO, lpxR*	This work
pELPR	pQLinkN containing *lpxE, pagL, pagP, lpxR*	This work
pEOPR	pQLinkN containing *lpxE, lpxO, pagP, lpxR*	This work
pFLPR	pQLinkN containing *lpxF. pagL, pagP, lpxR*	This work
pLOPR	pQLinkN containing *pagL, lpxO, pagP, lpxR*	This work

Table 5.2 Bacterial strains and plasmids used for chapter 3.

Strain or plasmid	Genotype or description	Source or reference
Strains		
W3110	Wild type, F^- I^- rph-1 INV(*rrnD, rrnE*)1 *rph-1*	*E. coli* Genetic Stock center (Yale)
BN5	W3110 Δ*eptA,* Δ*lpxT,* Δ*lpxM::kan*	156
L12M12	BN5 pQ-M_{YP}	This work
L12M14	W3110 Δ*eptA,* Δ*lpxT*	This work
L12M16	BN5 pQ-M_{CJ}	This work
L14M14	L12M14 *LpxL::tet* pQ-M_{VC}	This work
L16M14	L12M14 *LpxL::tet* pQ-M_{CJ}	This work
L14M12	BN5 *LpxL::tet* pQ-$L_{VC}M_{YP}$	This work
Plasmids		
pQLinkN	Vector containing a tac promotor, Amp^r	106
pACYC-M_{EC}	pACYC-LpxM_{EC}	This work
pQ-L_{CJ}	pQLinkN containing *lpxL* from *C. jejuni*	This work
pQ-L_{VC}	pQLinkN containing *lpxL* from *V. cholerae*	This work
pQ-M_{YP}	pQLinkN containing *lpxM* from *Y. pestis*	This work
pQ-M_{CJ}	pQLinkN containing *lpxM* from *C. jejuni*	This work
pQ-M_{HP}	pQLinkN containing *lpxM* from *H. pylori*	This work
pQ-$L_{VC}M_{YP}$	pQLinkN containing *lpxL* from *V. cholerae* and *lpxM* from *Y. pestis*	This work

5.2 PLASMID CONSTRUCTION AND GROWTH CONDITIONS

5.2.1 Plasmids and growth conditions of strains generated in Chapter 2.

Six genes, *lpxE, lpxF, lpxO, lpxR, pagL,* and *pagP,* were cloned into pQLinkN, as previously described[106]. Transformation of BN1 and BN2 with the plasmids yielded the 61 strains in chapter 2. All strains were grown at 37° C in Luria-Bertani Broth (LB) supplemented with 100 μg/ml ampicillin where appropriate and 50 μM to 1 mM IPTG with an optimized isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration between 50 μM and 1 mM, as determined by TLC analysis of enzyme activity.

5.2.1 Plasmids and growth conditions of strains generated in Chapter 3.

The *lpxM* genes from *Y. pestis* (GENE NAME) and *C. jejuni* (GENE NAME) and the *lpxL* genes from *V. cholerae (GENE NAME), C. jejuni*(GENE NAME), and *H. pylori*(GENE NAME) were cloned separately into the pQLinkN plasmid using standard cloning strategy. A combinatorial plasmid expressing *lpxL* from *V. cholerae* and *lpxM* from *Y. pestis* was generated as previously described. LpxM from *E. coli* was inserted into the tetracycline cassette of pACYC using standard techniques. See supplementary Table X for a full list of plasmids generated in this study.

All strains were grown at 37° C in LB supplemented with 100ug/ml ampicillin, 30ug/ml and 200 uM isopropyl β-D-1-thiogalactopyranoside (IPTG) where appropriate.

5.3 ISOLATION OF LIPID A

$^{32}P_i$ radiolabeled lipid A was isolated from 7 ml cultures for analysis by TLC as previously described[153158]. For MS, lipid A was prepared from 15 ml cultures[158,159] and analyzed using a MALDI-TOF/TOF (ABI 4700 Proteomics Analyzer) mass spectrometer in the negative ion linear mode as previously described[158].

5.4 TLR SIGNALING ASSAYS

The HEK-Blue™ hTLR4, HEK-Blue™ hTLR2, and THP1-XBlue™ -MD2-CD14 cell lines were purchased from Invivogen and maintained according to their specifications. Whole cell aliquots and LPS samples were serial diluted for assays as previously described[77,159]. At least two biological replicates were each done in triplicate and one representative set was shown here.

5.5 ISOLATION AND QUANTIFICATION OF LPS.

LPS was isolated from all strains by phenol extraction and purified by the Hirschfeld method as previously described[158,159]. Quantification was achieved using the 3-deoxy-D-*manno*-octulosonic acid (Kdo) colorimetric assay[160] to normalize the samples to 0.5 mg/ml using *E. coli* K12 LPS (LPS EK-Ultrapure, Invivogen) as a standard.

5.6 WHOLE CELL BACTERIAL SAMPLE PREPARATION

Cells for assays were prepared by growing a diluted overnight culture to an OD600 of 1.0 and washing with sterile phosphate buffered saline (PBS) to remove lysed cells or vesicle fragments. Cell pellets were gently resuspended in 5 mls of PBS, and the OD600 was measured. 5 x 10^9 cells were harvested by centrifugation, gently resuspended in 1 ml PBS and aliquoted for storage at -80° C. CFU plating after storage at -80° C confirmed equivalent cell counts between samples.

5.7 LIPOPOLYSACCHARIDE STIMULATION ASSAYS WITH THP-1 CELLS AND CYTOKINE ANALYSIS

THP-1 human monocytes (ATCC) were maintained according to ATCC's specifications. THP-1 monocytes were differentiated into macrophages and stimulated for 24 h with 100 ng/ml LPS as previously described[161]. LPS samples were quantified as described above and normalized to the number of LPS molecules. Culture supernatants

from triplicate wells were harvested and sent to Ocean Ridge Biosciences (Palm Beach Gardens, FL) for Luminex quantification of the cytokines: TNF-α, IL-1β, IL-6, IL-8, G-CSF, RANTES and MCP-1.

5.8 PURIFICATION OF INDIVIDUAL LIPID A SPECIES

Purification of 3-*O*-deacyl-4′-monophosphoryl lipid A produced by strain BN1/pELP was performed by reverse-phase chromatography as described previously[162] with ~ 0.4-0.6 mg of the target lipid obtained per liter of culture. The amount of purified lipid A was quantified by phosphate determination as previously described[163]. Based upon TLC analysis, approximately 1/3rd of the lipid A synthesized is 3-*O*-deacyl-4′-monophosphoryl lipid A. Assuming there are 10^9 CFU/ml of bacteria at an OD_{600} of 1.0 and ~10^6 lipid A molecules per cell[8], the maximum yield of the target lipid would be ~1 mg/L of culture.

5.9 MOUSE IGG AND CYTOKINE QUANTIFICATION

All procedures were performed according to IACUC and institutional guidelines. Monophosphoryl lipid A (MPLA) was purchased from Invivogen and is referred to as MPL, for simplicity. Female Balb/cJ mice (Jackson) (7 mice/group), 6-8 weeks old, were bled from the tail vein (~20 μl) prior to primary injection (day -1) and on day 28. Serum was stored at -20° C for further analysis. Emulsions were prepared as described previously[164], and on day 1 mice were immunized subcutaneously into the backpad with 50 μl of an emulsion of 30 ug lysozyme from chicken egg white (HEL) with 6 pM of purified lipid A, determined by phosphate quantification as previously described[163]. Secondary and final immunizations were performed intraperitoneally on day 21 and day 35, respectively, with 50 μl of the same emulsions. On day 36, serum was collected by heart puncture for Luminex cytokine analysis.

Serum IgG titers were determined by ELISA serial dilution. Serum dilutions from 1/200 through 1/437000 were captured on HEL coated high binding ELISA plates (Corning Costar 3590) and detected with 1/5000 anti-Mouse IgG HRP (Jackson Immuno, 115-065-209). Plates were analyzed with GraphPad Prism software and titers were determined as the point where the non-linear fitted curve determined 3-fold signal above background.

5.10 BACTERIAL GROWTH IN THE PRESENCE OF ANTIBIOTICS AND MEMBRANE STRESSORS

Growth assays were performed in LB broth at 37° C in a 96-well plate format. Each well contained 150 ul of inoculum at OD_{600} 0.05 containing increasing concentrations of one of the following compounds at the levels described in the figures: ethylenediaminetetraacetic acid (EDTA) (Fisher), bile salts (Sigma), sodium dodecyl sulfate (SDS) (Fisher), and Vancomycin (Fisher).

5.12 STATISTICAL ANALYSIS

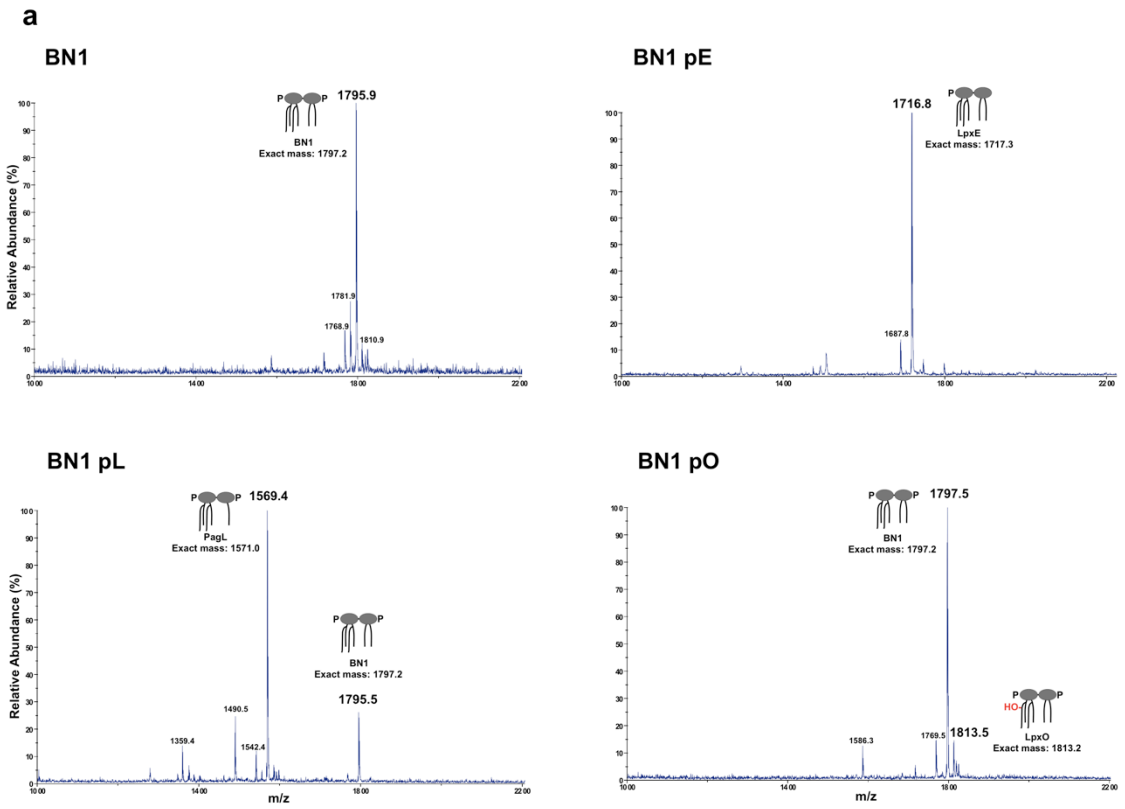
Statistical analysis was performed using one-tailed T-tests. P-values were calculated with an $n \geq 3$, $\alpha = 0.05$ or 0.01, as reported in the for TLR, THP-1, and membrane susceptibility assays. Error bars on graphs refer to standard deviation.

Appendices

Appendix A. Mass spectrometry results for all strains used in chapter 2.

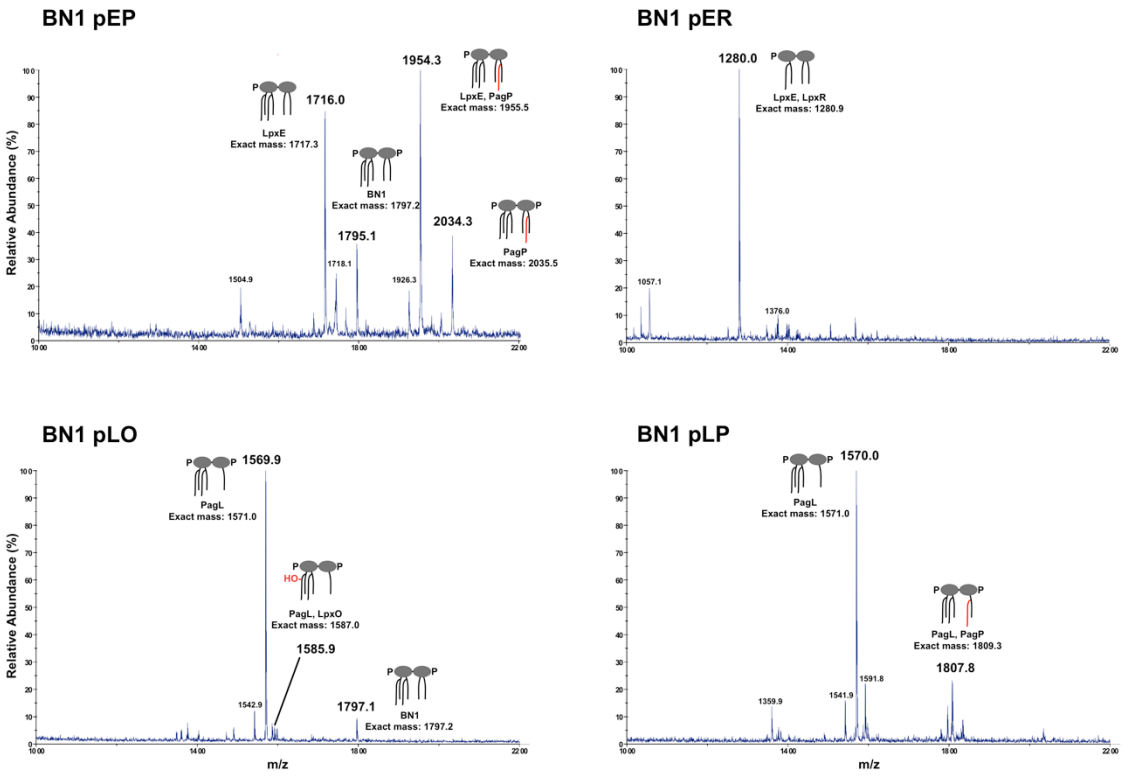
All spectra, excluding the 3 examples presented in the main text, can be found in this figure. Lipid A structures corresponding to the mass peak are depicted by cartoons next to the peak. Peak clusters at *m/z* ~1375 correspond to phospholipid contamination, confirmed by TLC isolation of the species. The labile 1-phosphate can be lost, resulting in a mass difference of ~ 80 mass units. a,b) Negative ion mode MS of BN1 and BN2 strains, respectively, confirmed the activity of the enzymes expressed in combinations. A minor species of penta-acylated lipid A can be observed in some enzyme combinations, corresponding to a peak at *m/z* ~1585. c) Positive ion mode MS was done for all strains expressing both phosphatases, LpxE and LpxF. Positive mode often results in single or double sodium adducts on the molecules, resulting in peak masses that are ~23 or 46 mass units higher than the exact mass of each structure.

Group A. BN1 strains.



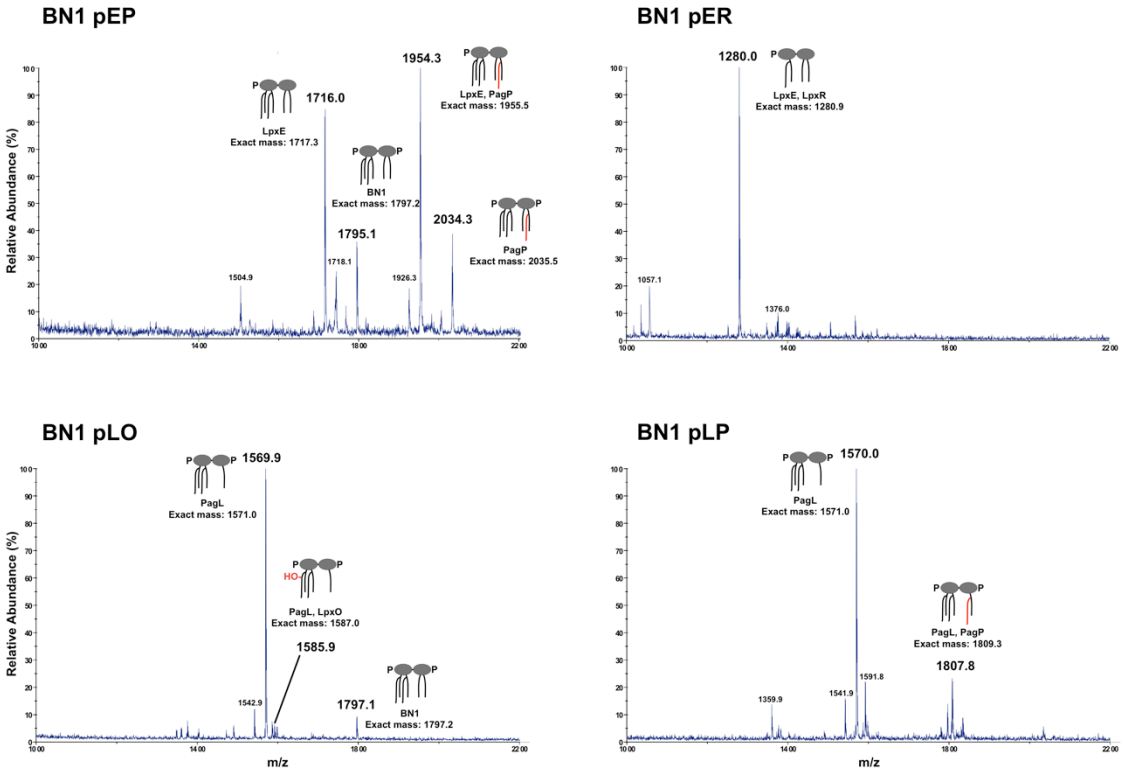
Group A continued. BN1 strains.

a, cont'd



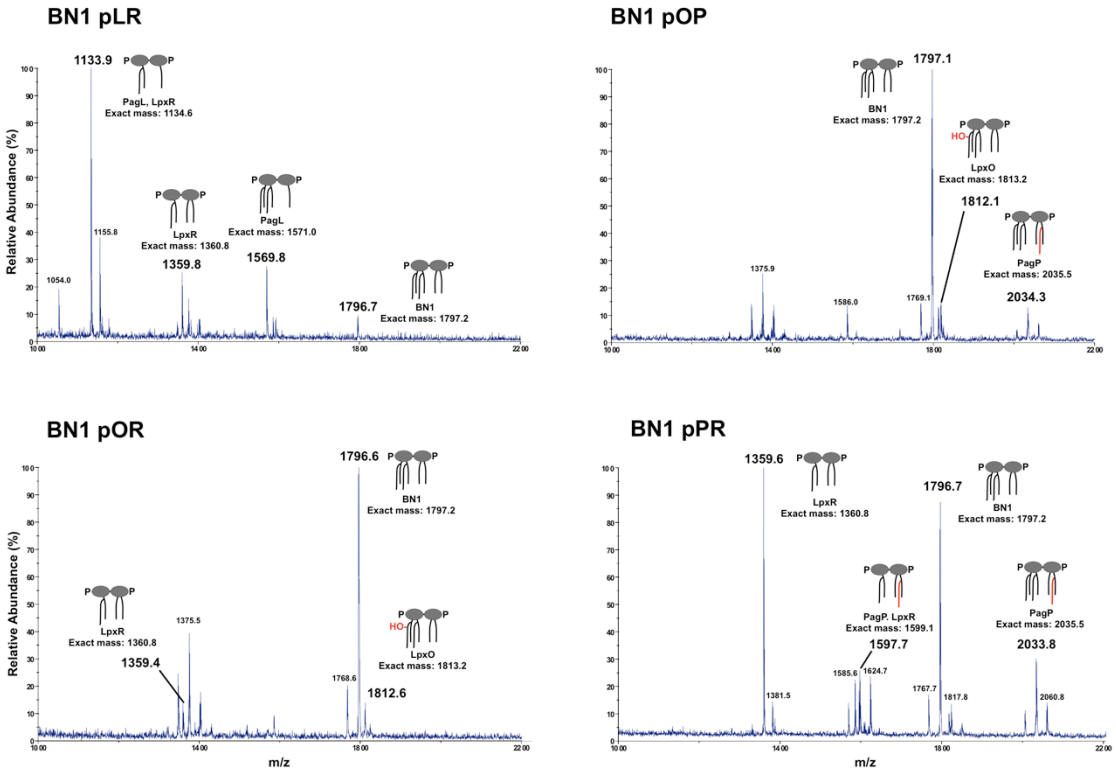
Group A continued. BN1 strains.

a, cont'd



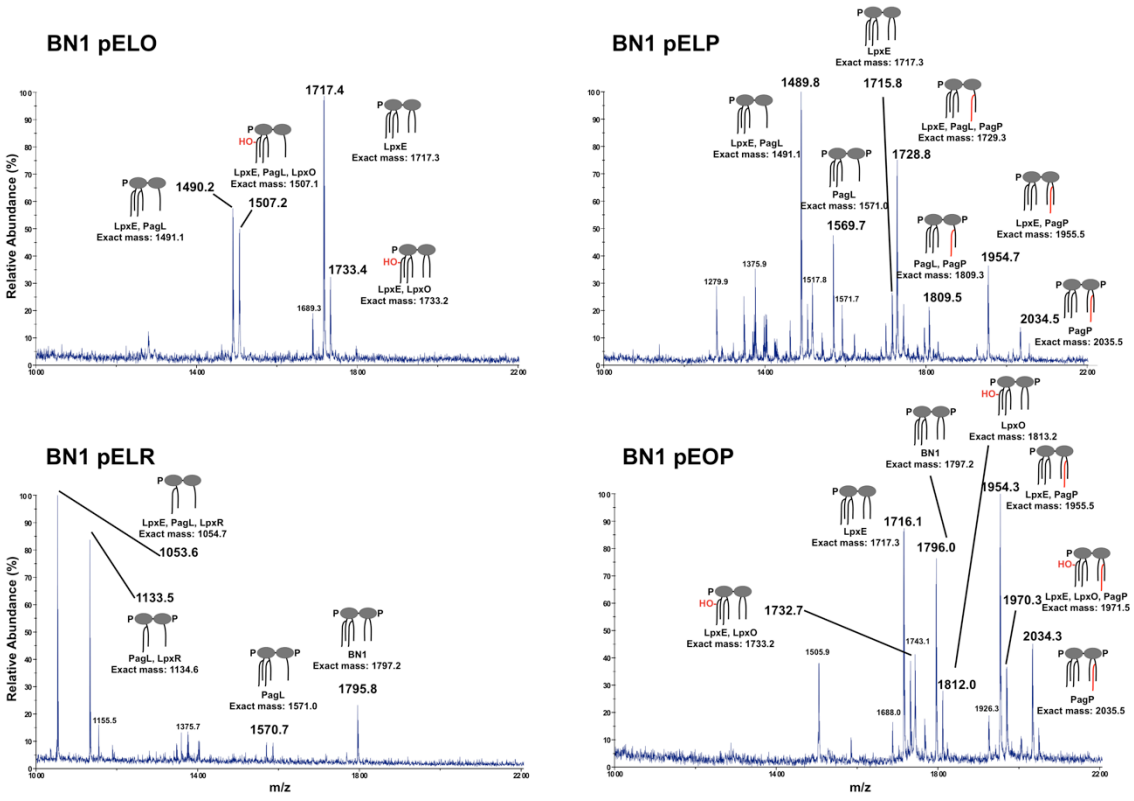
Group A continued. BN1 strains.

a, cont'd



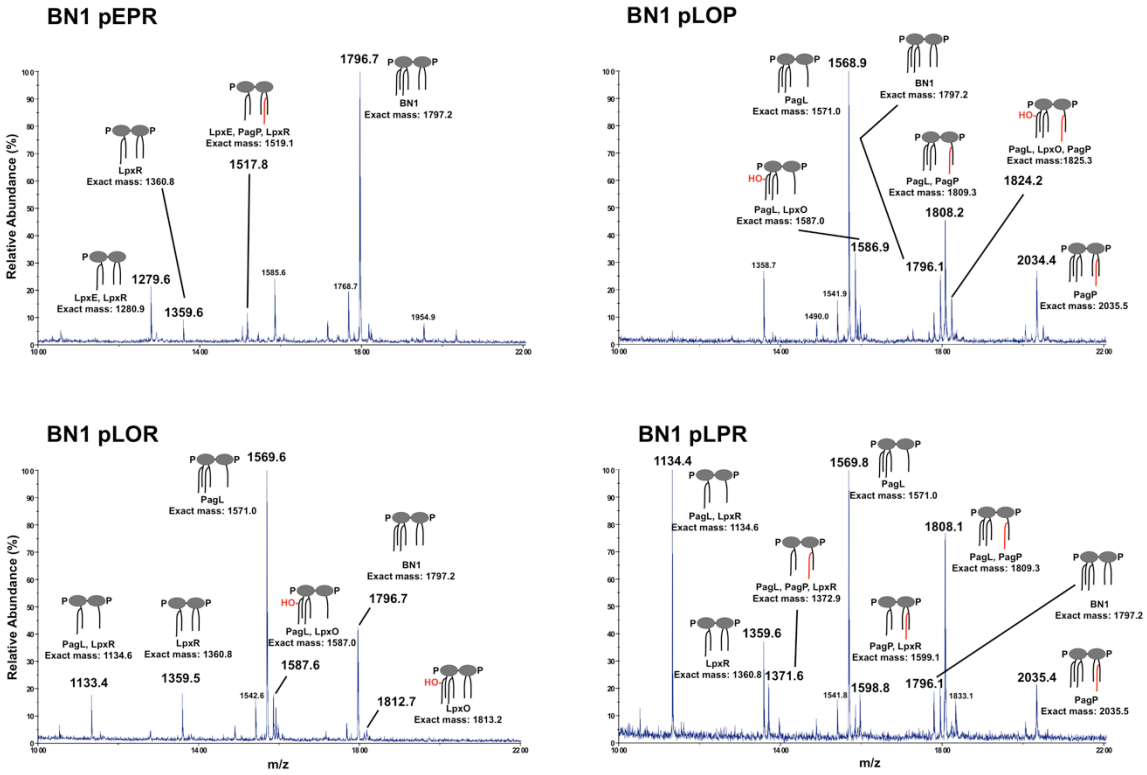
Group A continued. BN1 strains.

a, cont'd



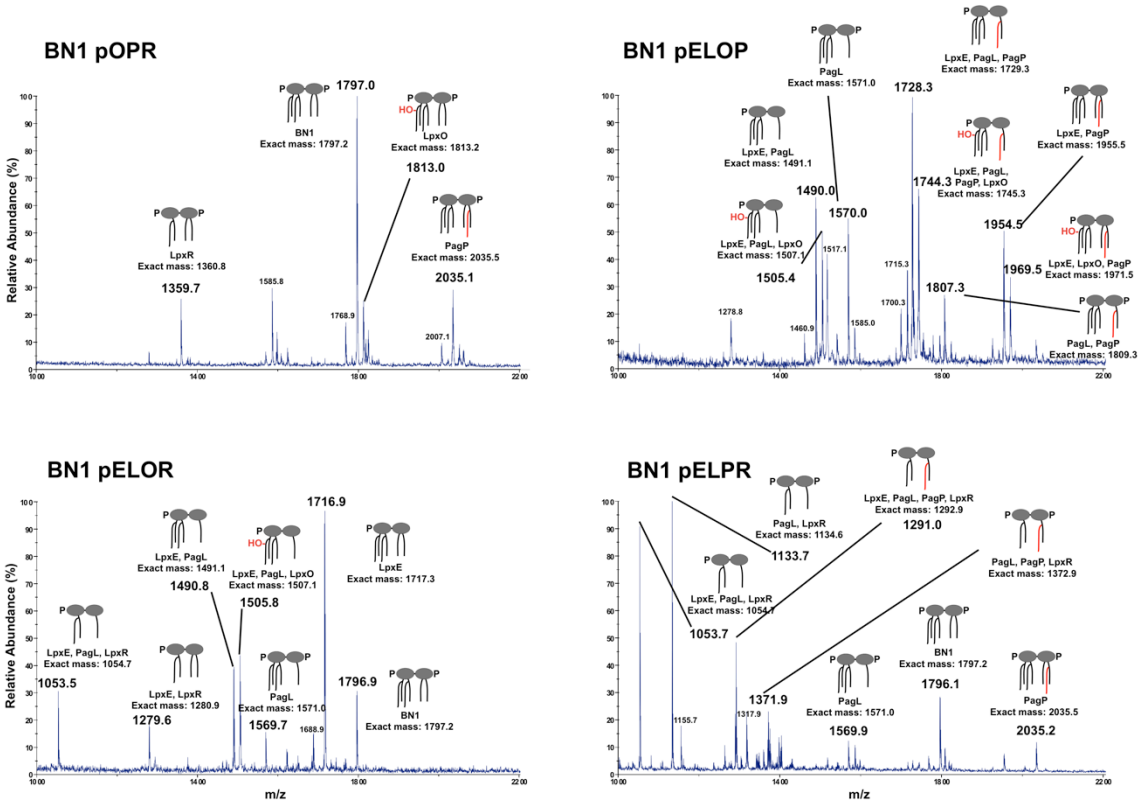
Group A continued. BN1 strains.

a, cont'd



Group A continued. BN1 strains.

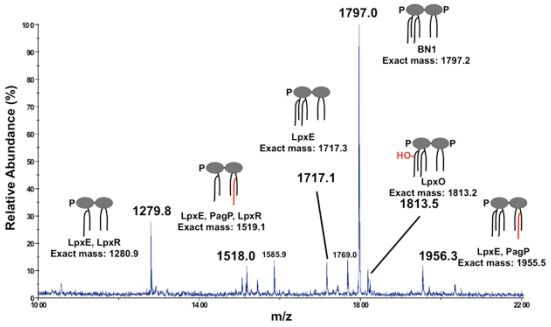
a, cont'd



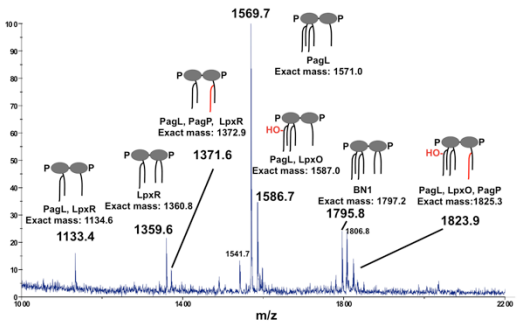
Group A continued. BN1 strains.

a, cont'd

BN1 pEOPR

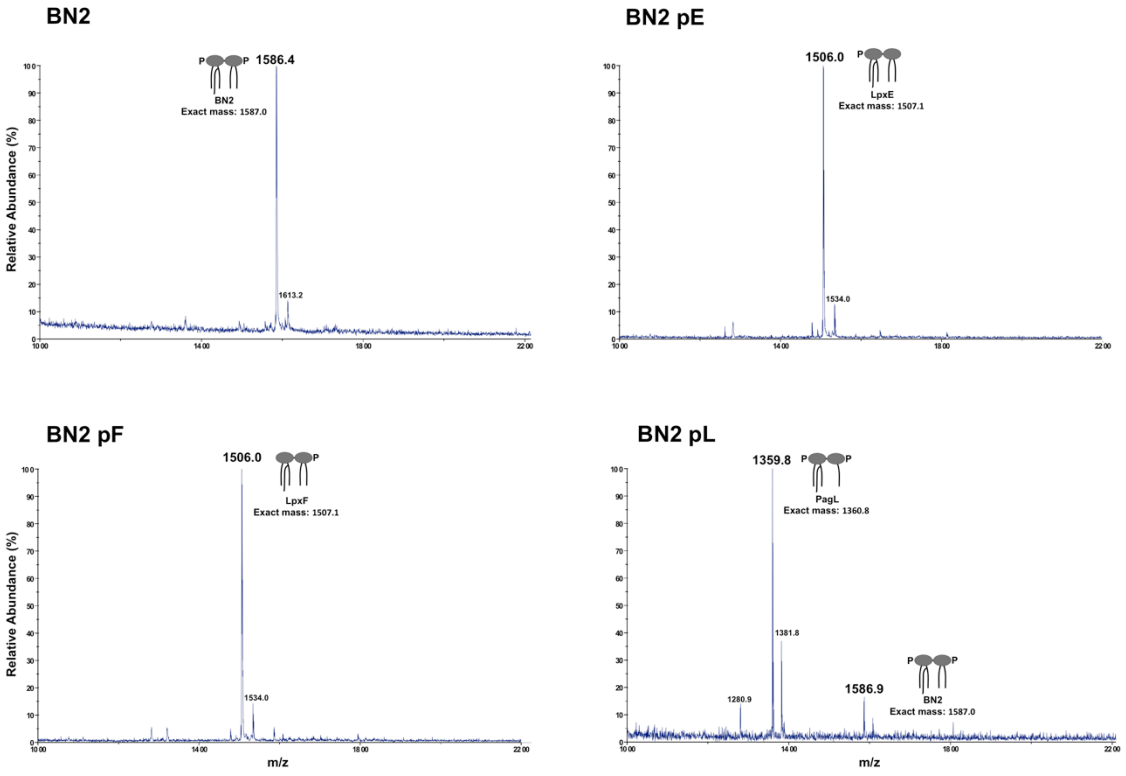


BN1 pLOPR



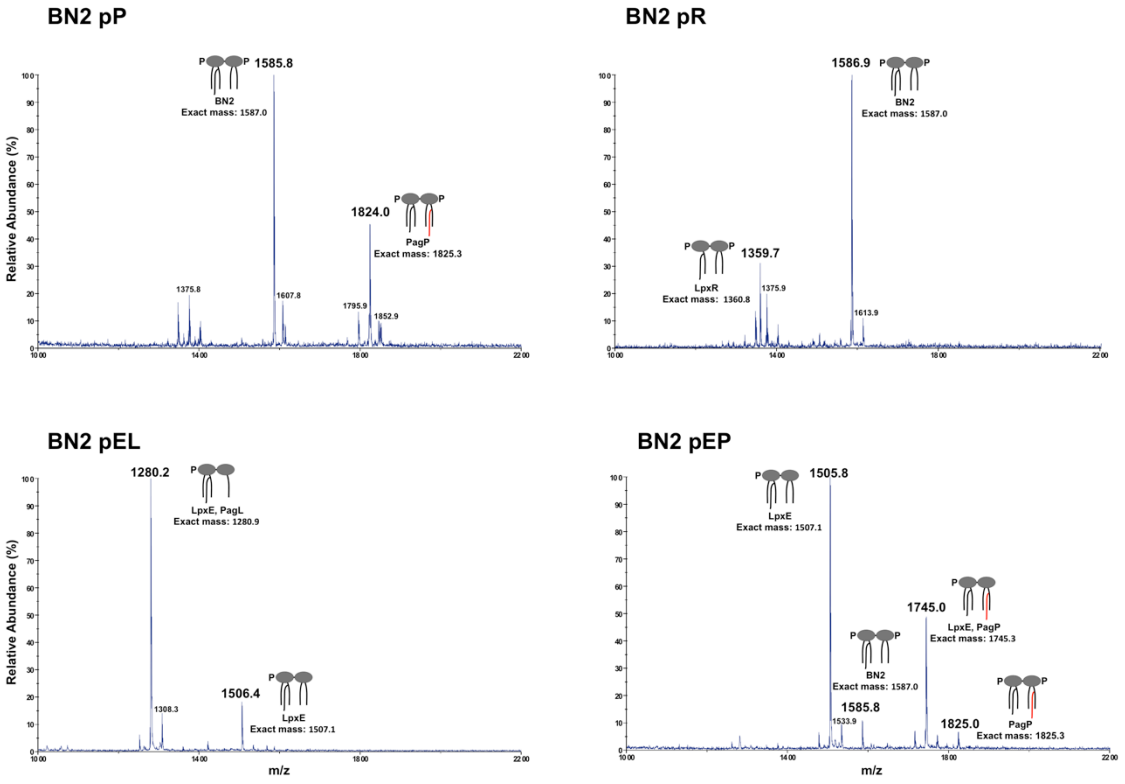
Group B. BN2 strains.

b



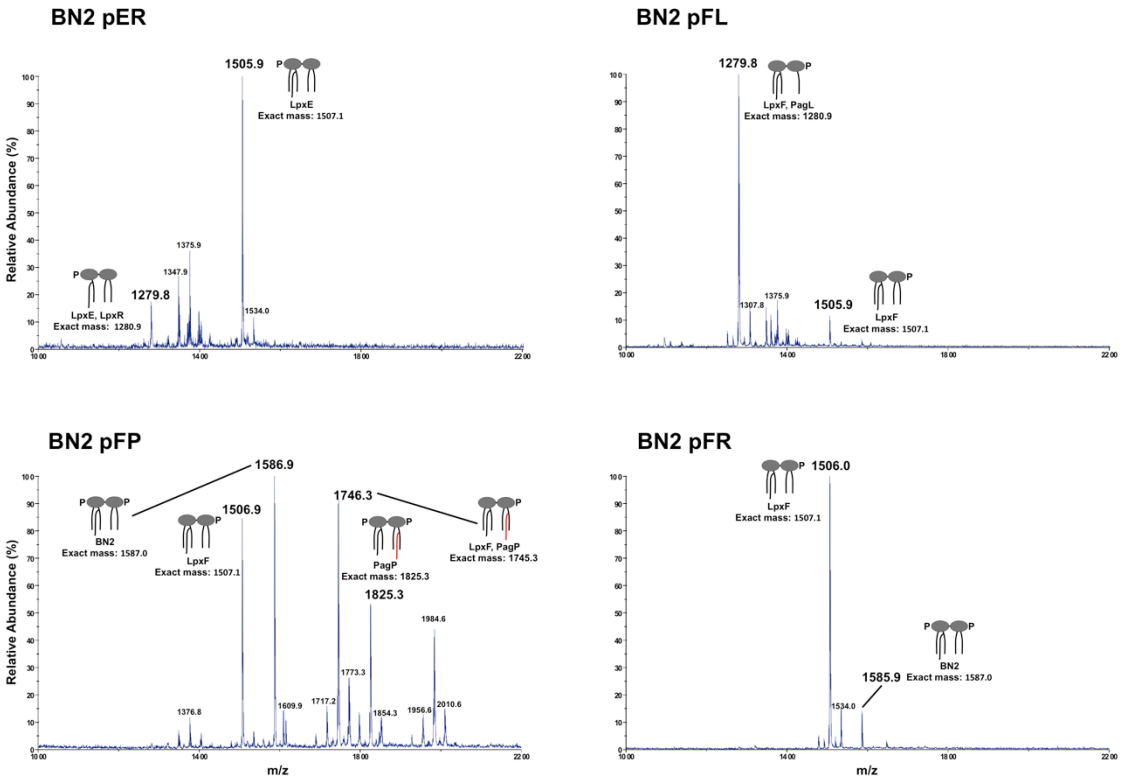
Group B continued. BN2 strains.

b, cont'd



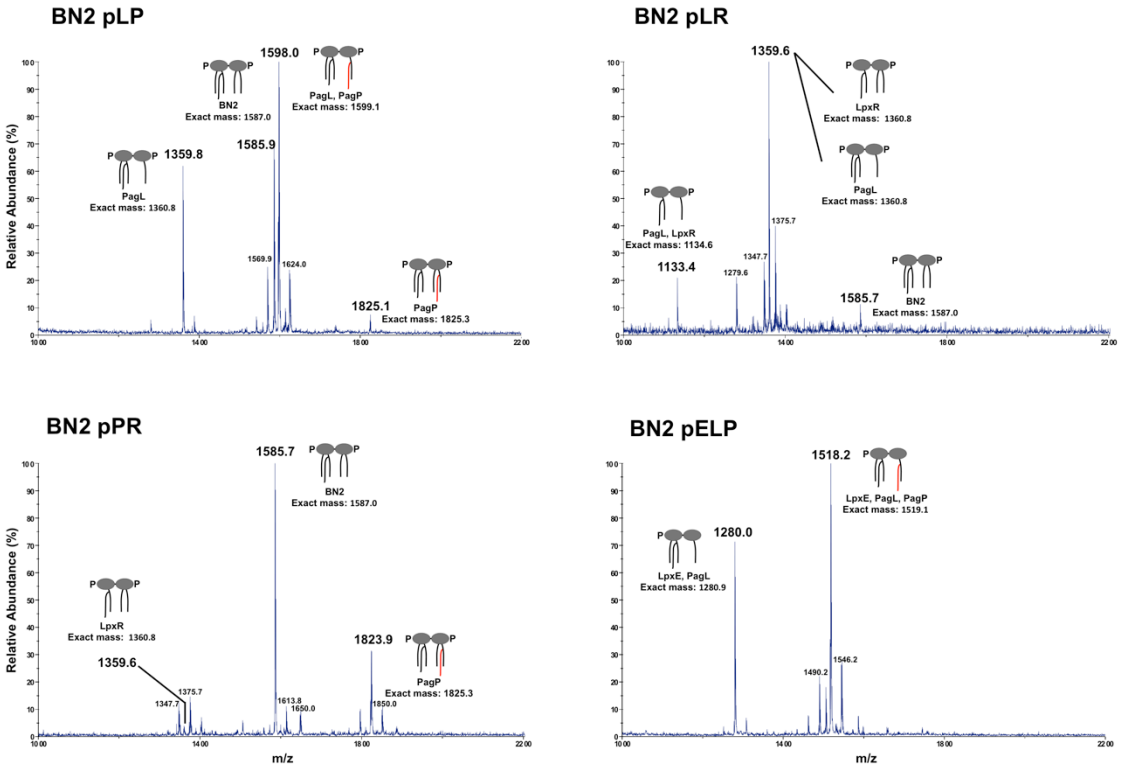
Group B continued. BN2 strains.

b, cont'd



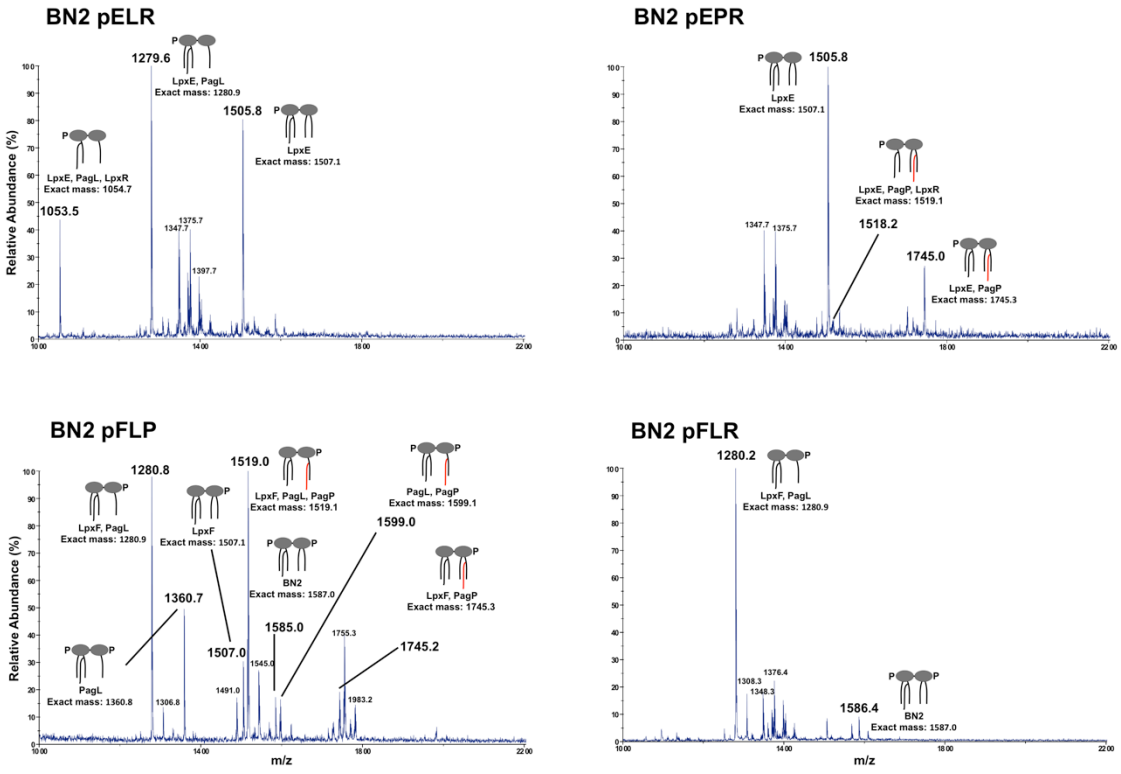
Group B continued. BN2 strains.

b, cont'd



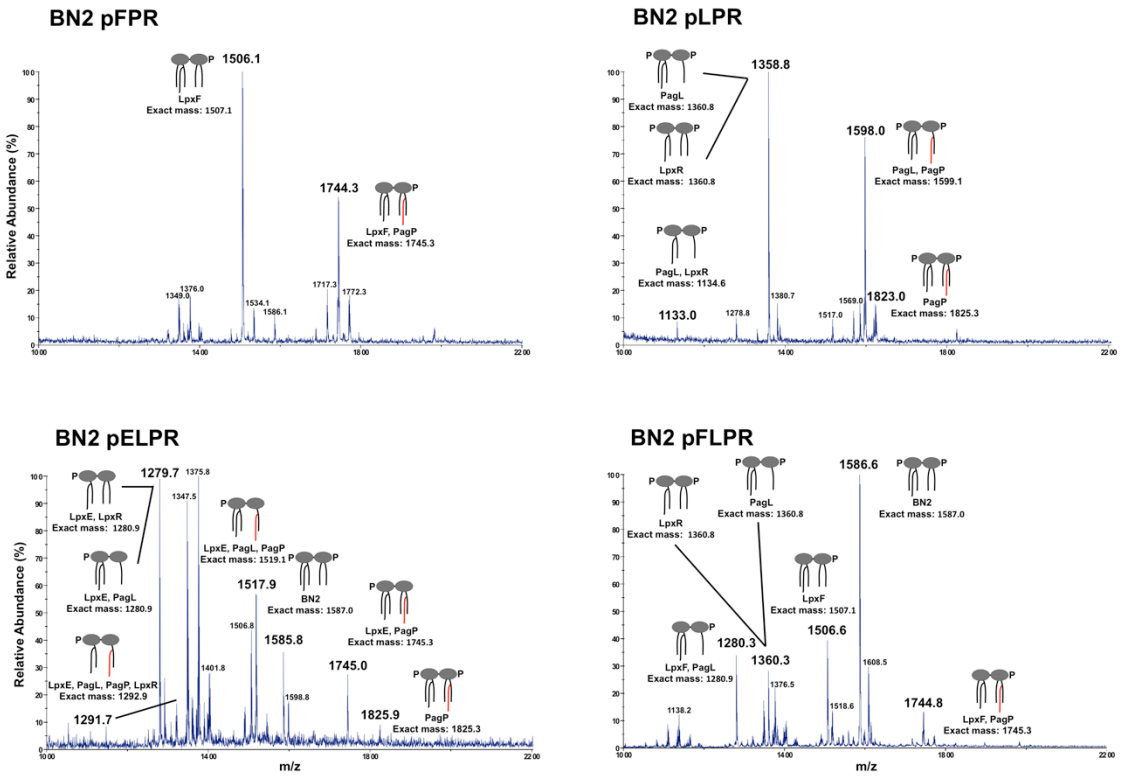
Group B continued. BN2 strains.

b, cont'd



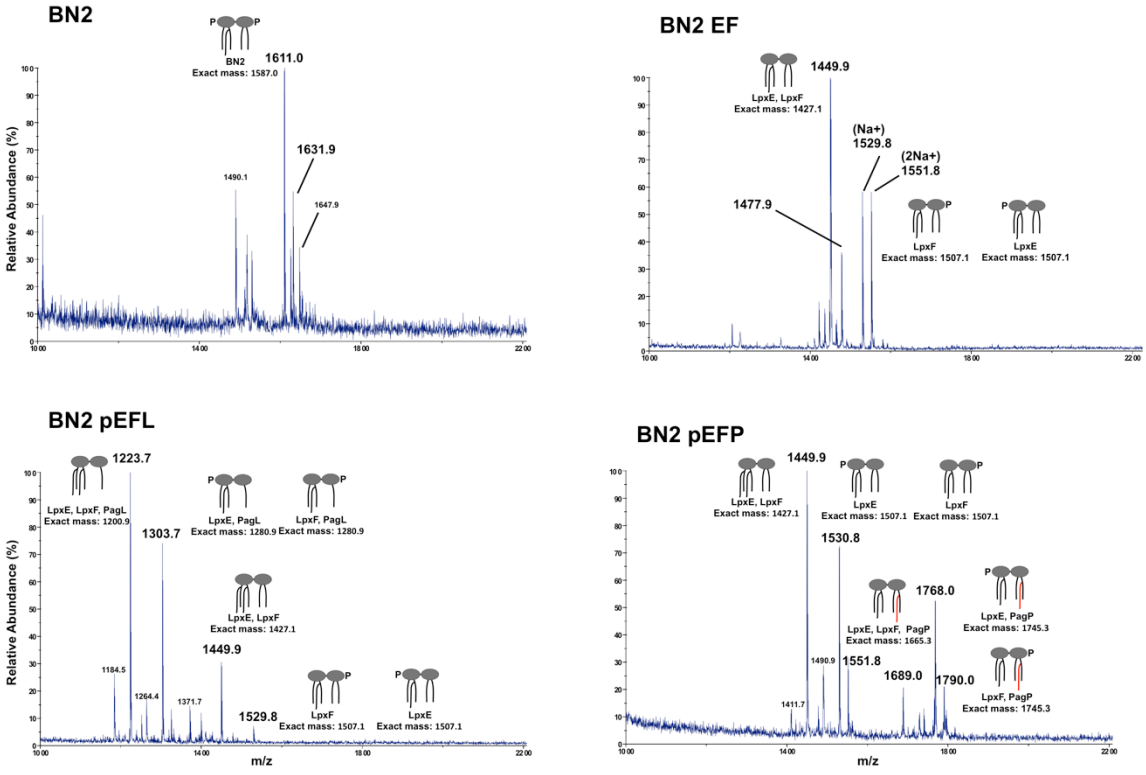
Group B continued. BN2 strains.

b, cont'd



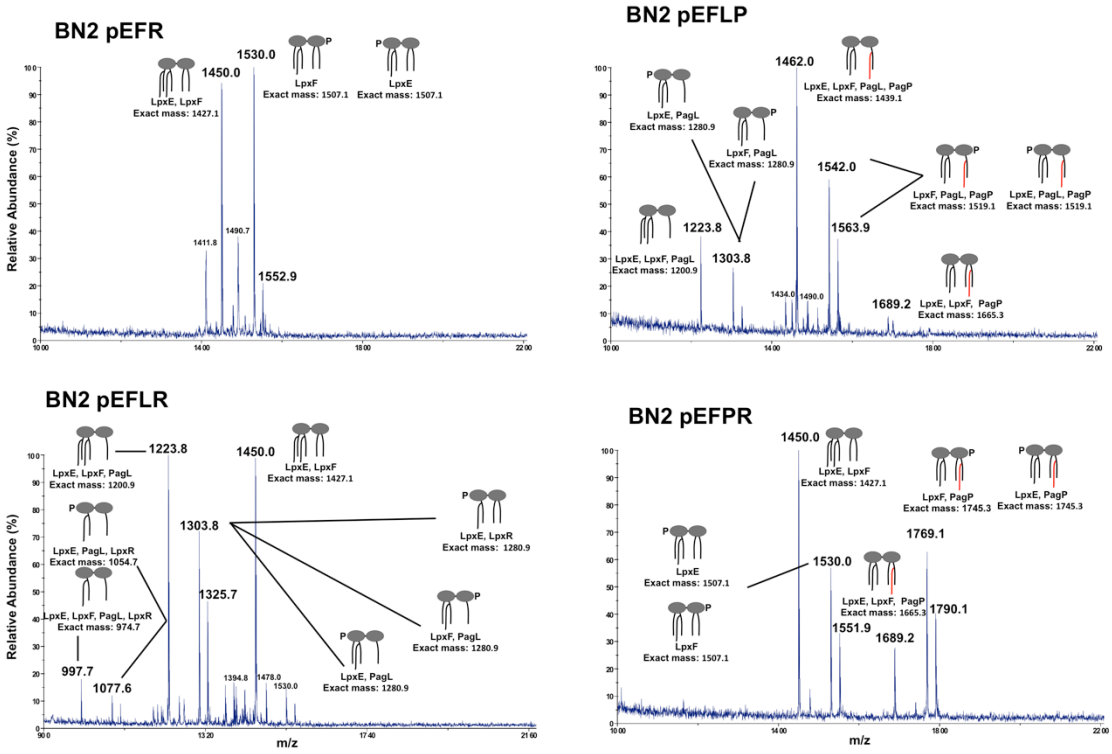
Group C. Positive mode analysis.

c



Group C continued. Positive mode analysis.

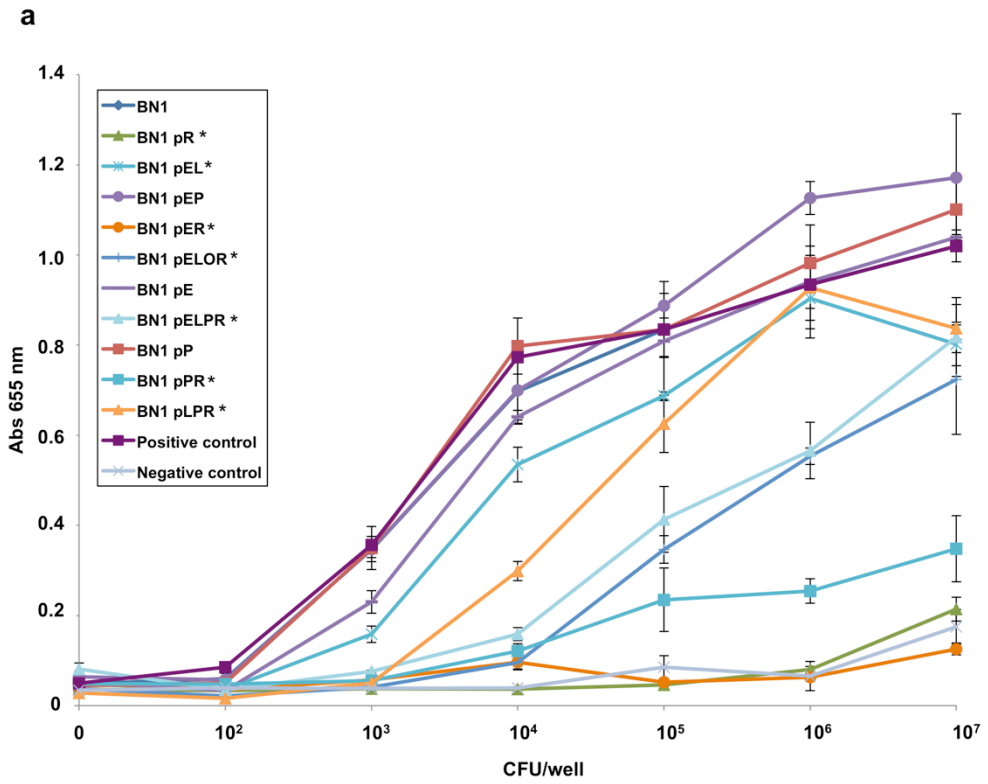
c, cont'd



Appendix B. Quantitative data represented colorimetrically in Fig. 2.6

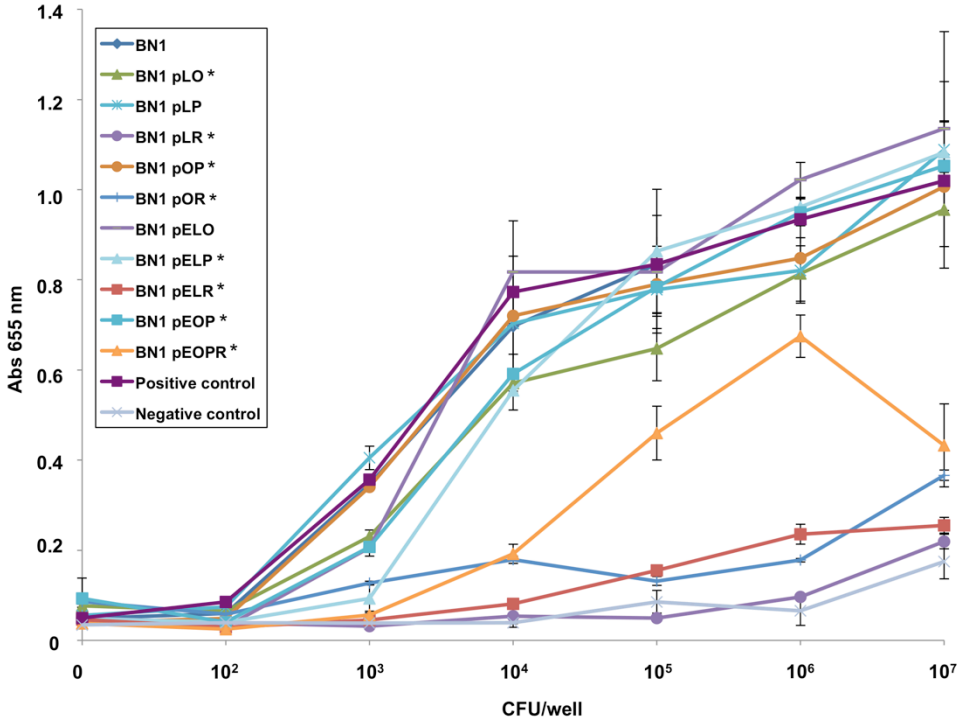
All TLR4 data used to generate the colorimetric scale presented in Figure 2c is graphed here. Significance is indicated by an asterisk and the p-values for samples that are significantly different from the BN1 background strain are denoted. a) TLR4 stimulation is shown of all strains in the BN1 background. These are split into three graphs due to number of samples. b) TLR4 stimulation is shown of all strains in the BN2 background, split into three graphs due to number of samples. c) TLR4 stimulation with LPS is shown.

Group A. BN1 strains.



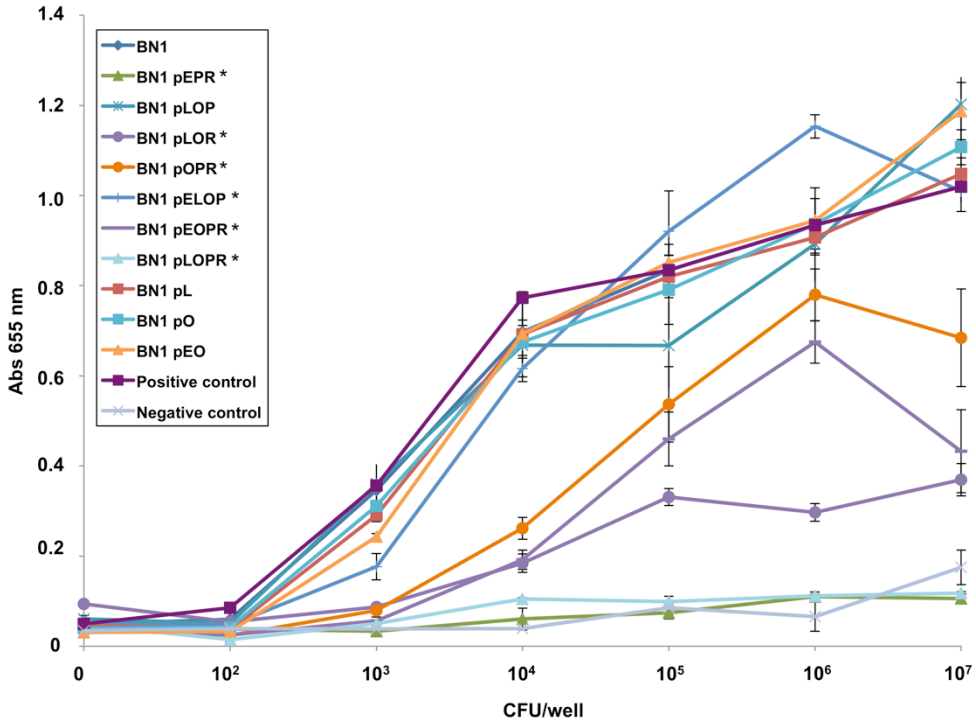
Group A continued. BN1 strains.

a, cont'd



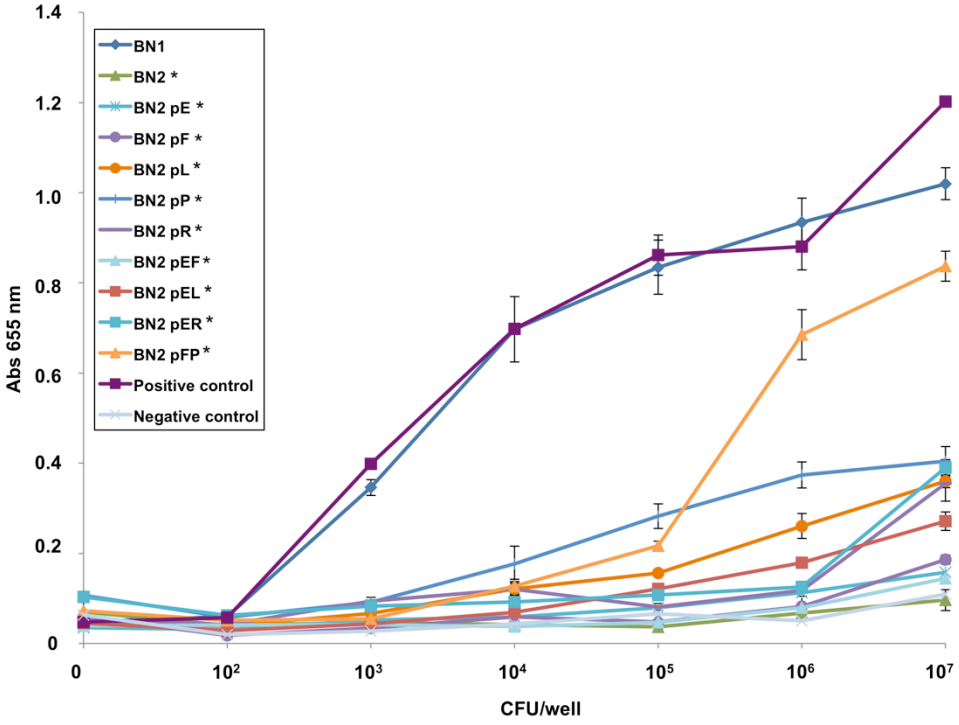
Group A continued. BN1 strains.

a, cont'd



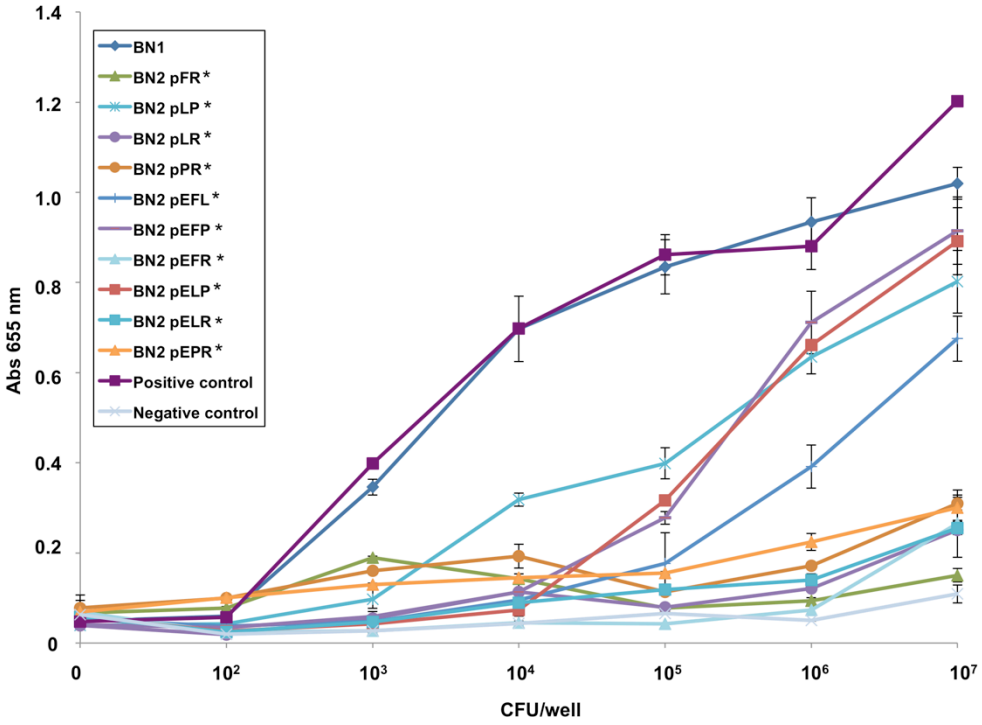
Group B. BN2 strains.

b



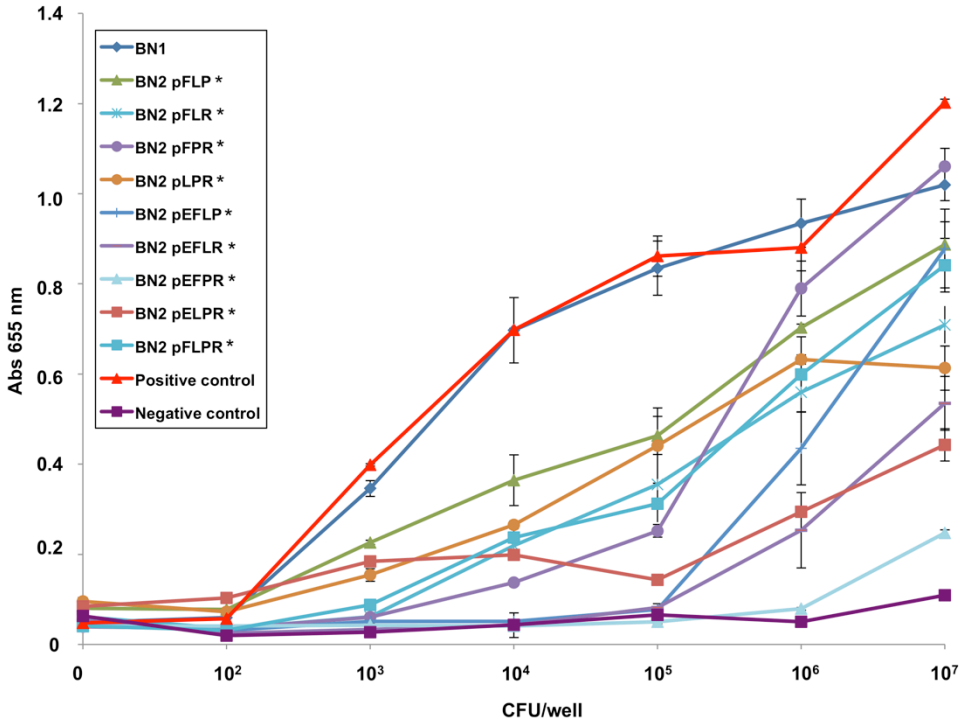
Group B continued. BN2 strains.

b, cont'd



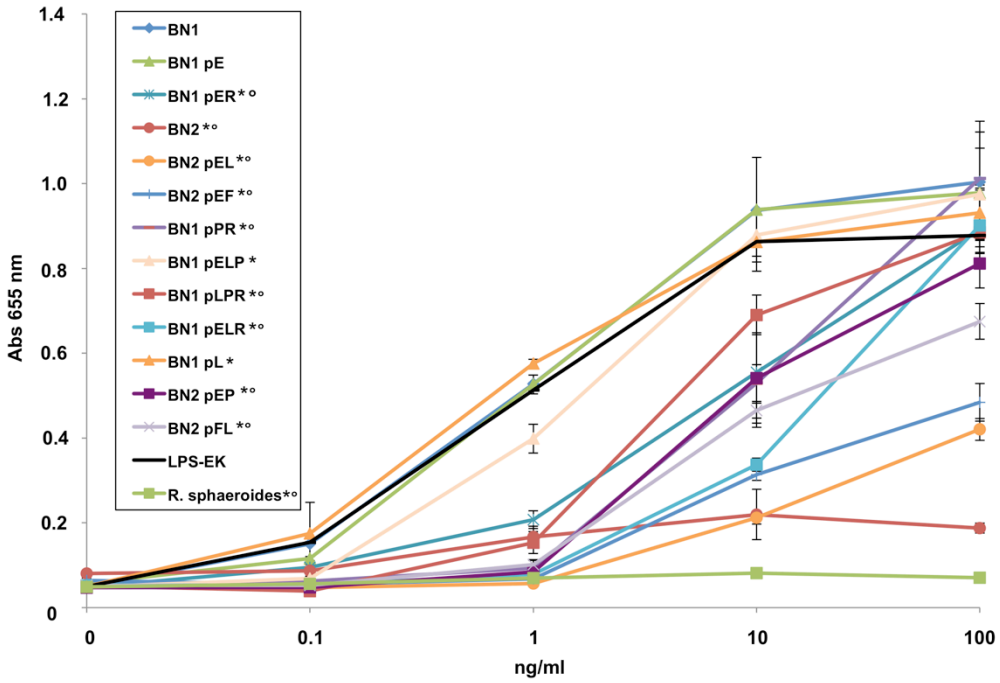
Group B continued. BN2 strains.

b, cont'd



Group C. LPS stimulation.

c



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